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The main goal of this project is to culture blood stem cells in the presence of human hematopoietic growth factors to produce autologous megakaryocytes (MK) to be transfused into patients as a supplement to the conventional stem cell autograft. The purpose of the clinical trial is to determine whether the transfused MK will generate platelets in vivo in great enough numbers to reduce or eliminate the need for repeated platelet transfusions following high-dose chemotherapy and autologous stem cell transplant. We determined in pre-clinical experiments that a cocktail of the growth factors thrombopoietin, IL-3 and Flt3-L and high seeding cell density were necessary to optimize the ex vivo expansion of MK. We successfully transplanted three patients. Cell expansion was optimal after six days, no negative reactions were observed, and the patients recovered normal platelet levels fast with normal platelet function. This study shows that using an ex vivo expanded product is feasible, and the successful outcome in our three transplanted patients augurs well for future use of this concept. Additionally, we have added a novel survival factor, PLP-E to the arsenal of growth factors used for ex vivo myeloid expansion and have elucidate the molecular mechanism of proplatelet formation.

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INTRODUCTION:

The primary objective of this project is to culture blood stem cells in the presence of human hematopoietic growth factors to produce autologous megakaryocytes (MK) to be transfused into patients as a supplement to the conventional stem cell autograft. The purpose of the clinical trial is to determine whether the transfused MK will generate platelets *in vivo* in great enough numbers to reduce or eliminate the need for repeated platelet transfusions following high-dose chemotherapy and autologous stem cell transplant. Although they decrease the risk of fatal bleeding complications, repeated platelet transfusions increase the risk of bacterial and viral infections, alloimmunization and transfusion reactions.[1] When this study was initiated, no FDA-approved megakaryocyte-specific growth factors were available for clinical use. We therefore had to resort to growth factors being developed by pharmaceutical companies, and seek FDA approval for their use in this study.

As described in our previous reports, the study was plagued throughout with pitfalls originating from the pharmaceutical industry canceling on more than one occasion their prior approval for using their products. Often this happened following several months of painstaking laboratory investigation with results published in scientific publications. As a result, we were not able to adhere to the timeline described in the Statement of Work. Our initial grant proposal was based on growth factors provided by GD Searle & Co. (Promegapoietin (PMP) and Progenipoietin (PGP)[2]), which was later acquired by Pharmacia, which terminated the PMP/PGP growth factor development program.

We then repeated our pre-clinical laboratory investigations[3] with the use of Megakaryocyte Growth and Development Factor (MGDF) from Amgen, which, in combination with Stem Cell Factor (SCF), yielded an excellent growth factor cocktail.[4] Unfortunately, Amgen decided to remove MGDF from clinical development.

With considerable effort, we were able to generate similar preclinical data using a growth factor combination obtained from a variety of sources including Immunex (Flt3L) and R&D Systems, Inc. (thrombopoietin -TPO- and IL-3).[5] Our study was revised, an IND submitted and approved by the FDA, and our first patient entered. The results obtained were very encouraging with full platelet recovery occurring at day 6 post-transplant. This unusual rapid recovery must be attributed to the *ex vivo*-expanded cells. Midway into processing the expanded megakaryocytes for our second patient, we were notified that the FDA was no longer permitting the use of the TPO obtained from R&D Systems because of concerns regarding the purification process. This new obstacle caused us to abort the culture for the second patient. This new concern on the part of the FDA has affected cellular therapy research at many other centers.

We then found an alternative source of clinical grade TPO manufactured by Pharmacia using purification procedures approved by the FDA. In the meantime, Amgen acquired Immunex and then Pfizer acquired Pharmacia. These reorganizations caused further delays with respect to licensing issues surrounding our use of Flt-3L and TPO, including considerable time required to draft contracts between Amgen, Pfizer, and our Office of Research and Sponsored Projects. Finally, the accrual of potential candidates has also been hampered by either the restrictions that we had established in our clinical trial protocol, the consent of the patient, and diminished willingness of health insurance providers to allow their customers to go on study, due to their reduced risk tolerance in the wake of falling financial returns.

We have decided to stop the clinical trial in view of the termination of our funding period. All in all, of the six patients who were accrued, three patients were transplanted successfully. While the megakaryocytes expanded optimally for the other three patients, they could not be transplanted for reasons discussed in the Body description. This study showed that using an *ex vivo*-expanded product is feasible and the successful outcome in our three transplanted patients augurs well for future use of this concept.

While our translational project was stalled, we also investigated means to improve expansion of human MK in vitro and determined the signaling mechanism of platelet production.

BODY:

Materials and Methods Used for Pre-Clinical Trial (summary from annual reports 01 to 03).

Low density nonadherent mononuclear cells were prepared from bone marrow and peripheral blood progenitor cells. CD34+ cells were purified using immunomagnetic procedures. For small scale cultures, an average of 90.7% of cells were viable with an average purity of 81%. For large scale cultures an average of 82% cells were viable with an average purity of 97% as assessed by flow cytometry. The cells were cultured at a density of 4x10E5 cells per ml of X-vivo-20 medium supplemented with a cocktail composed of thrombopoietin (TPO) and non-specific fast proliferating cytokines (IL-3 and Flt3-L). The expansion of early and late MK was monitored by flow cytometry and clonogenic assays.

Materials and Methods Used for Clinical Trial (spanning annual report 04 and last one) [6]

Equipment

- 37° water bath, Scientific Products
- Class B sterile tissue culture hood, Baker Co.
- Clinical bench centrifuge, Beckman.
- 37° tissue culture incubator, 5% CO_2, Forma Scientific.
- Variable adjustable electronic pipettor, EDP, Rainin.
- Vacuum-driven automatic pipet device, Drummond.
- Coulter Epics XL 3-color laser flow cytometer, BeckmanCoulter Corp.

Materials

- Sterile pipet tips with aerosol barrier.
- Sterile individually wrapped polypropylene transfer pipets.
- 2ml, 5ml, 10ml, 25ml sterile individually wrapped polystyrene pipets with aerosol barrier.
- Sterile centrifuge tubes, Corning.
- 1cc, 3cc, 10cc, 20cc, 30cc, and 60cc sterile syringes, Becton Dickinson.
- 18G blunt nose needles, Becton Dickinson.
- Teflon tissue culture bags, 1L, (270ml nominal capacity) and 7ml capacity, Fluoroseal.
- Plastic volume-limiting pinch clips.
- Sterile Luer-Lok Connector tubing and bottle top septums, Nalgene.

Reagents

- Culture Medium: Sterile GMP-grade X-Vivo 20 serum-free medium, BioWhittaker, Walkersville, MD
- Heparin: Preservative-free, Life Technologies.
- Dnase I: Roche Diagnostics. Reconstituted with sterile water and filtered through a
- Thawing Medium: X-Vivo 20 supplemented with 10 IU/ml heparin and 10μg/ml Dnase.
- rhuTPO, GMP grade, Pharmacia (previous sources for TPO and TPO-related growth factors were from R&D, Amgen, Searle).
- rhuIL-3, GMP grade, R&D Systems, Minneapolis, MN. 25 μg per vial, reconstituted with 250μl sterile PBS
- Flt-3L, GMP grade, Immunex Corp, Seattle WA. 250 μg per vial, reconstituted with 250μl sterile water

  20μL aliquots of each cytokine stored at -70°C in sterile tubes until needed. Thawed aliquots of cytokines were kept at 4°C for up to two weeks.
- 1% Trypan Blue (Sigma) in saline.
- Phycoerythrin-cyanin 5.1 (PC5) conjugated-α-CD34, Clone 581, Beckman Coulter,
- Phycoerythrin (PE) conjugated -α-CD41 Clone P2, Beckman Coulter
- Fluorescein isothiocyanate (FITC) conjugated-α-CD15, Clone 80H5, Beckman Coulter
- Clonogenic assay kits for CFU-MK (MegaCult C) and CFU-GM (MethoCult), Stem Cell Technologies, Vancouver, BC, Canada
- Quantikine huTPO ELISA, R&D Systems

Specimen Requirement

- Cells from a ten liter leukapheresis to be selected for CD34+ cells using the Clinimacs system. The CD34+ purified cells were then frozen by the Bone Marrow Transplant laboratory using the standard cell freezing system.

Procedure

A. Sample Preparation

1. Frozen CD34+ cell tube placed into a resealable plastic bag. Close bag (to prevent any water seepage into tube).

2. The bag with the tube is then placed in a 37° water bath and gently agitated until the cell suspension has thawed to a small ball of ice (approximately 3-4 minutes per 5 ml tube).

3. The bag is wiped dry and transferred to a Class B sterile tissue culture hood.

4. In the sterile tissue culture hood, the contents of each tube of thawed cells is transferred to a sterile 50ml centrifuge tube using a sterile polypropylene transfer pipet and diluted 1:10 by drop-wise addition of

cold thawing medium using a sterile polypropylene transfer pipet with very gentle agitation/vortexing.

5. The tube is sealed and the cell suspension is centrifuged at 260xg for 10 min, 4°.

6. The cell pellet is resuspended in 2-3 ml X-Vivo 20 using a sterile polypropylene transfer pipet, then diluted to 50ml with X-Vivo 20 by decanting fresh medium. A 50µl sample for cell concentration analysis is taken using a sterile pipet tip with aerosol barrier. This sample is diluted 1:1 with Trypan Blue, applied to a hemacytometer, and the cell concentration and viability determined using a phase contrast microscope.

7. The cell suspension is centrifuged again at 260xg for 10 min at 4°. The cell pellet is resuspended in 2-3ml of X-Vivo 20 using a sterile polypropylene transfer pipet, then diluted with up to 20ml X-Vivo 20 by decanting fresh medium.

B. Culture Preparation

1. The final volume (FV) required to achieve a cell concentration of 400,000 cells/ml is calculated using the cell count obtained above in A.6.

2. The bag's nominal volume is adjusted with bag clip barriers so as to contain a volume sufficient to suspend the cells at 400,000 c/ml at an average fluid height of 1cm.

3. The cells are injected using a 20cc syringe into a 1L Teflon bag attached via Luer-Lok connections.

4. X-Vivo 20 and sufficient cytokines to achieve a final concentration of 100ng/ml Flt-3L, 100ng/ml TPO and 10ng/ml IL-3 are then added using a 60cc syringe attached via Luer-Lok connections to the bag to reach the FV calculated in B.1.

5. After gently agitating the bag to resuspend the cells, a 3ml aliquot is removed using a 10cc syringe. This is injected into a 7ml Teflon bag, adjusted with bag clip barriers so as to contain a volume sufficient to suspend the cells at an average fluid height of 1cm. This test sample is used to assay the culture progress and avoid opening the main sample bag until adjustment is necessary (See Sample Assays below). Both bags are stored in a 37° tissue culture incubator at 5% CO_2 and >90% relative humidity used solely for the purpose of clinical patient sample incubation.

1. Each day a 100µl of culture is removed from the test sample bag using a 1 ml syringe for cell concentration measurement. If the cell concentration is in excess of 800,000 c/ml, then a new bag clip barrier is placed on the bag so as to contain the new nominal volume required to dilute the cells to 400,000 cell/ml, and the old bag clip barrier is removed. The required volume of fresh X-Vivo 20 w/ cytokine

cocktail is added to the main sample using a 60cc sterile syringe attached via Luer-Lok connections to the bag. If the new total volume exceeds the 1cm liquid height capacity of the bag, a new bag is attached to the old bag via a Luer-Lok connector tube, and the sample is equally divided between the two bags by suspending the bags until the volumes are equilibrated by gravity. Bag clips are used to keep the volume height at the specified 1cm.

C. Sample Assays

The following assays are performed at the start and end of the culture: Samples are washed and stained for phenotypes CD34, CD41, and CD15 and analyzed by flow cytometry.

1. 100,000 cell aliquots are washed in 1% BSA (Sigma) in PBS (GIBCO) with 5mM EDTA (Sigma), designed to prevent further platelet activation and/or reverse adherence of activated platelets. The wash buffer is aspirated and the pellet resuspended in 50µl of PBS.

2. After washing, the cells are stained for 15 min at 4°C in the dark with 5µl of PC5-CD34, PE-α-CD41, and FITC-α-CD15 and analyzed by flow cytometry on the same day. The negative controls are PC5-, PE- and FITC-α-mouse IgG_1 used at equivalent IgG_1 concentrations. Only the non-apoptotic high forward scatter, low side scatter cell population was used for subset analysis.

3. Clonogenic assays are performed according to the manufacturer's instructions. Viable cells from each day of assay are seeded at a concentration of 10^3 cells/ml. CFU-MK are scored after ten days and CFU-GM/BFU-E after fourteen days.

4. Twenty-four to forty-eight hours prior to the culture endpoint, a small aliquot of the patient cell culture is assayed for the following: Quantitative PCR for malignancies other than breast cancer and histochemistry for breast cancer cells. Sterility: Bacterial, viral, and fungal contamination, endotoxin titer, and mycoplasma levels.

5. When the designated culture period is reached, the sample bag(s) is(are) attached via Luer-Lok connector tubes to a cell washing bag and transfer to a COBE 2991 Cell Washer. Cells are prepared for transplant according to Bone Marrow Transplant Laboratory procedures.

6. A small aliquot of the buffer from each round of washed cells is assayed for residual TPO by ELISA, following manufacturers instructions, to monitor effectiveness of washing at removing cytokines from cell suspension.

Materials and Methods Used for Investigating the role of murine prolactin-like protein E on enhancing the expansion of human megakaryocytes in vitro:[7]

The PLP-E cDNA was linked in-frame to a secreted alkaline phosphatase (AP) gene in a mammalian expression vector. Fusion protein was obtained by transient transfection of the DNA construct into Chinese hamster ovary cells and subsequent collection of culture medium over a 2-day period. Medium containing secreted AP-PLP-E was concentrated and used in binding assays. PLP-E without the AP fusion partner was generated by similar approaches. Other methods described above.

Materials and Methods Used for Investigating the molecular mechanism of proplatelet formation:[8]

This investigation was carried out with the human CHRF-288 megakaryoblastic cell line.

Cell Culture: CHRF-288 cells were cultured in Fischer's medium supplemented with 20% fetal bovine serum as a source of growth factors and integrin ligands. To identify the serum factor responsible for MK differentiation, serum-free culture was carried out with X-vivo-20 medium in plates coated with various extracellular matrix proteins. PMA at 10 ng/ml was used to promote cell differentiation.

Ploidy analysis: Nonadherent cells were collected by centrifugation. Adherent cells were dislodged with 0.05% trypsin in 0.33 mM EDTA, and the viable cells counted by trypan blue exclusion. Then, 5X10E% cells per sample were washed in PBS containing 1% BSA. DNA was stained with 7-amino-actinomycin D following one-step fixation-permeabilization with the Ortho-Permafix reagent. Ploidy classes were then determined following flow cytometric analysis.

Protein extraction and Western Blot: Cells were collected after PMA treatment. Nonadherent and dislodged adherent cells were centrifuged at 400g for 5 min and the cell pellets lyzed in a buffer containing 30 mM Hepes, 100 mM NaCl, 10 mM benzamidine, 1 mM EDTA, 1% Triton-X-100 and 20 mM NaF, adjusted to pH 7.5. The following protein and phosphatase inhibitors were then added: 1 mM phenyl methyl sulfonyl fluoride, 10 ug/ml aprotinin, 5 ug/ml leupeptin, 2 ug/ml pepstatin, 1 mM Na vanadate. Cell lysates were cleared by centrifugation at 16,000 rpm for 5 min at 4°C. Lysates were denatured by boiling for 5 min in Laemmli sample buffer and loaded at a concentration of 20 ug per lane on 10% Tris-glycine iGel. Antibodies against signal proteins or phosphorylated signal proteins were used to determine the activation of the kinases during cell differentiation. Each antibody was diluted in blocking buffer and incubated with the blot at room temperature for 2 hours, then incubated with alkaline phosphatase conjugate for 1 hour at room temperature and color detected by BCIP/NBT substrate liquid system.

Other methods described above.

Results:

1.Small Scale Culture using cytokine combinations from various corporate sources (year 01-03)[2, 3, 5, 9]

The combination of Promegapoietin/Progenipoietin (Searle) resulted in increased frequency of MK progenitors (CD34+/CD41+) from 3.2% a day 3 to 14-19% at day 3 ($p<0.001$). Cultures seeded at high density (HD, $4x10^5$ cells/ml) resulted in improved expansion of MK progenitors and differentiated MK when compared to low seeding

density (LD, $1x10^5$ cells/ml) ($p<0.001$). By day 13, MK expansion per CD34+ cell reached 745-fold in HD cultures, compared to 500-fold in LD cultures. The combination of MGDF/SCF (Amgen) induced similar expansion of MK progenitors and differentiated MK. Since MK maturity at the time of transplant may influence the rate of platelet engraftment, we evaluated this parameter by grouping CFU-MK colonies on the basis of four size populations. Immature CFU-MK were present at days 0 and 3 at levels of 45% and 27%, respectively, and dropped to less than 5% at day 6 and even less or non-existent at day 9. The time of cell harvest determines, therefore, the stage of MK differentiation. We then substituted TPO, IL-3 (R&D Systems) and Flt3-L (Immunex) for the former growth factors, after MGDF and SCF became unavailable. The combination of 100 ng/ml TPO and Flt3-L and 10 ng/ml IL-3 proved effective for expanding CD34+/CD41+ cells. After the lengthy procedure for obtaining approval of a revised protocol by the Army Human Subject Board, we submitted a proposal to the FDA for an IND pertaining to a clinical protocol.

2. *Large Scale Culture (year 04-05) [5, 6]*

CD34 cells were cultured under optimal conditions as determined by the small scale experiments with the use of 100 ng/ml TPO, 10 ng/ml IL-3, and 100 ng/ml Flt-3L. Up to 270 ml were cultured in 1 L Teflon bags and assayed daily for 9 days. Small scale cultures indicated that 9 days of culture was required for adequate progenitor cell expansion, while at 6 days there were still a significant proportion of early MK progenitors indicated as essential for platelet engraftment. Thus, analysis will focus on results at Day 6 and Day 9. Cell expansion and viability was measured (Figure 1). Viable cell expansion was found to drop during the first 1 to 2 days of culture, then begin expanding after 3 days. Starting at Day 6 began expanding rapidly. Total viable cells expanded 4.5-fold, with 64% viability after 6 days and 11.7-fold with 69% viability after 9 days. However, there was little improvement in total cell viability after Day 4.

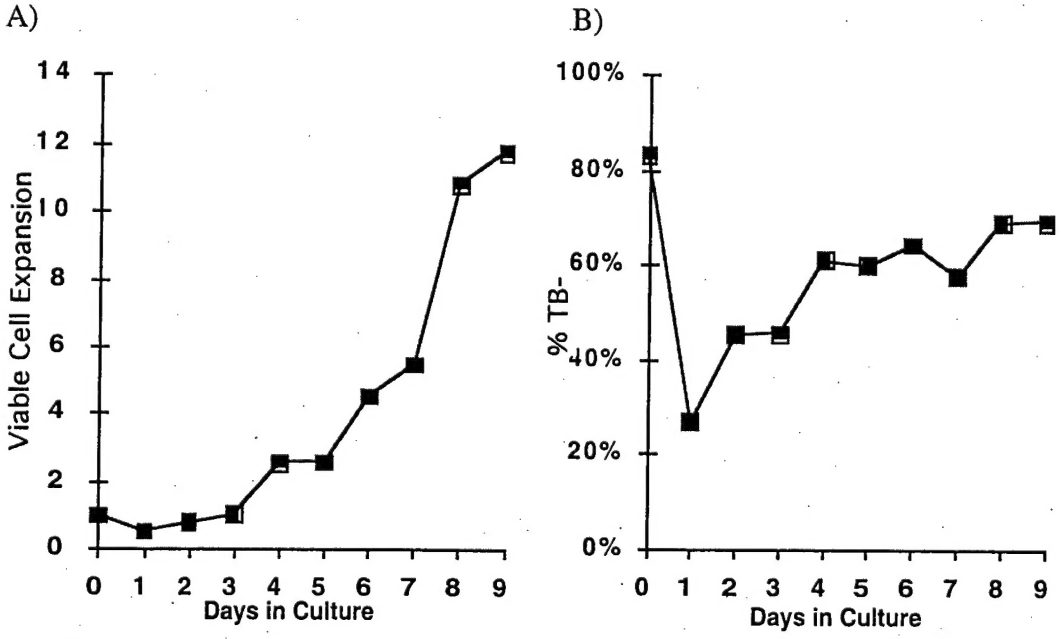

Figure 1: A) Total cell expansion and B) overall cell viability. Cells were assayed daily for number and frequency of Trypan Blue-negative cells.

Progenitor cell expansion

Progenitor frequency was assayed and relative expansion was calculated (Figure 2). $CD34^+$ cells declined to 59.9% of total cells at day 6 and 31.3% at day 9, but expanded

2.85-fold and 4.1-fold at day 6 and 9, respectively. Megakaryocyte progenitors comprised 13.2 and 13.4% of cells at day 6 and 9 respectively, and each seeded $CD34^+$ cell produced 0.6 and 2.1 $CD34^+/CD41^+$ cells after 6 and 9 days in culture. Megakaryocytes comprised 17.2% and 39.9%% of cells at day 6 and 9 respectively, and each seeded $CD34^+$ cell produced 0.8 and 6.2 $CD41^+$ cells after 6 and 9 days in culture.

Colony assays were performed after 3, 6 and 9 days and compared to seeded progenitors (Figure 3). CFU-GM and BFU-E colonies expanded over 9 days, and CFU-GEMM expanded slightly. CFU-MK numbers decreased at 3 days, then recovered by Day 9.

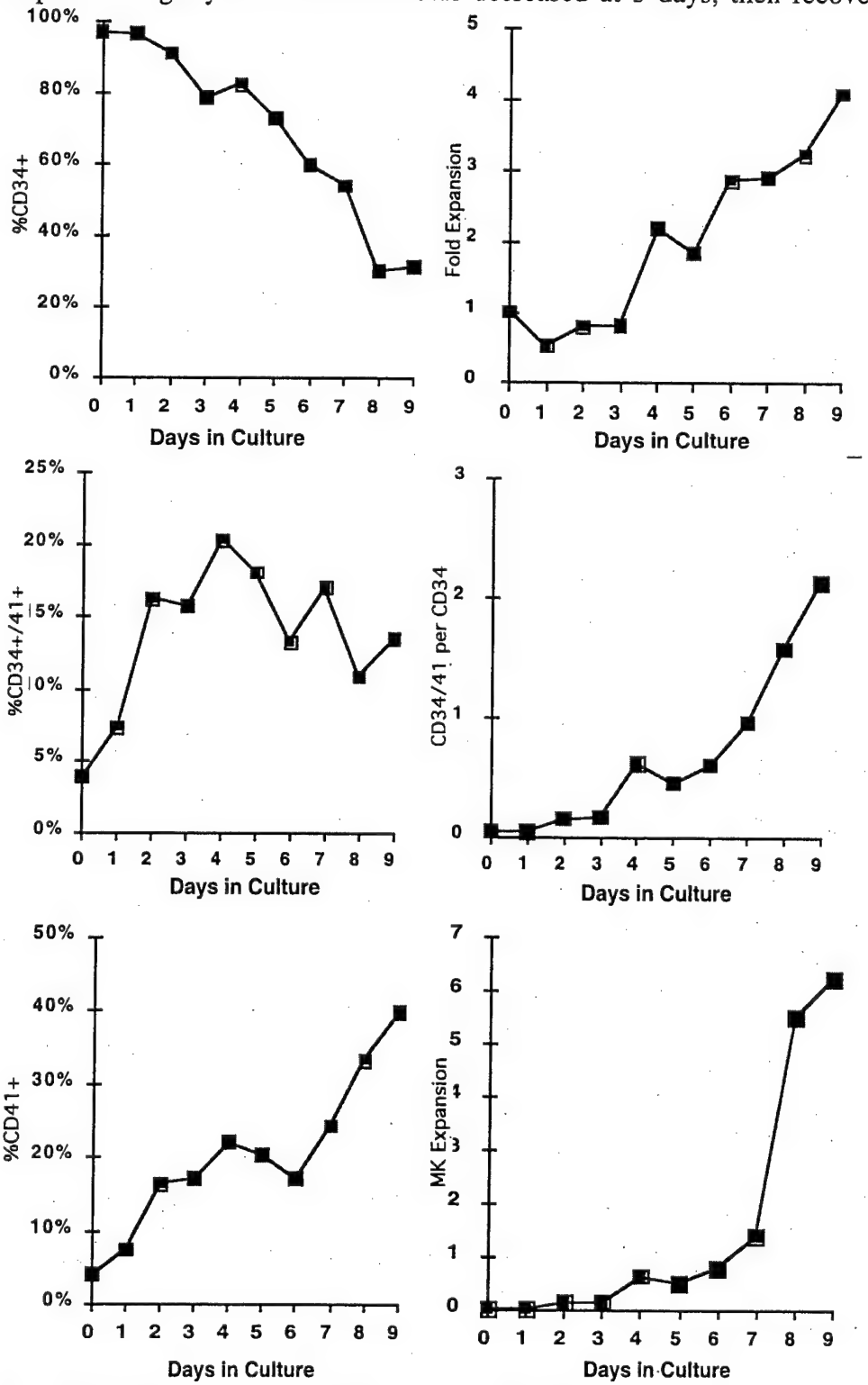

Fig. 2: Relative frequency and Expansion of CD34+, CD34+/41+, and CD41+ progenitor cells.

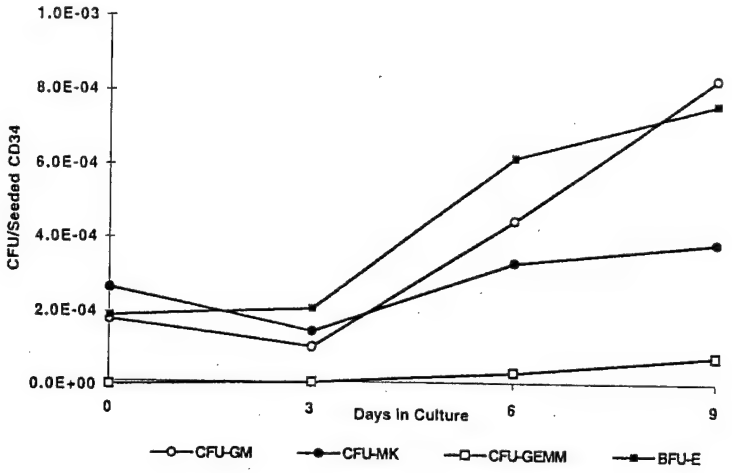

Fig. 3. Progenitor colony assay

3. Clinical Trial

Three patients were successfully transplanted. Patient NU97B2001 was transplanted with cells expanded using the cocktail of growth factors thrombopoietin and IL-3 from R&D and Flt3-L from Immunex at concentrations of 100 ng/ml, 10 ng/ml and 100 ng/ml, respectively. Platelet engraftment, defined as the day platelet count increases past 20,000/μl, occurred at day 6 post-transplant (26,000/mm^3) (Fig. 4). While expanded cells for the next patient were in preparation, the FDA notified us to avoid using thrombopoietin from R&D because of concerns regarding the purification process. Transplantation was aborted for this patient. Thrombopoietin from Pharmacia was substituted for the R&D product for the other two transplanted patients. Platelet engraftment for patients NU97B2004 and NU97B2005 occurred at days 11 and 8. The data relative to the transplant of the three patients is described in Table 1.

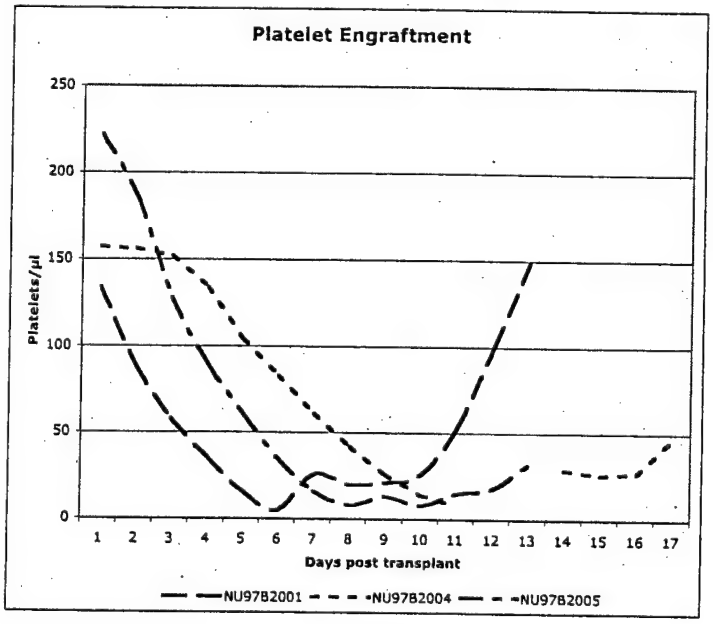

Fig.4.

Two additional patients selected for using the ex-vivo expanded product did not receive it. In one case, the cell viability of 58% was below the allowed theshold of 70% and in the other case, the endotoxin test was positive.

Table 1. NU97B2 Patient Sample Results

	Sample	NU97B2001	NU97B2002	NU97B2003	NU97B2004	NU97B2005	NU97B2006	Mean (n=6)
Cell Expansion Results	Culture Length (days)	6	9	9	6	6	6	**7**
	Infusion Date	10/30/01	3/4/02	4/2/03	4/16/03	4/23/03	8/21/03	
	Total Cells Seeded	1.04E+08	7.76E+07	5.11E+08	4.20E+08	8.61E+07	2.08E+08	**2.34E+08**
	Total Cell Expansion (Fold)	7.6	2.2	2.5	2.8	3.9	4.0	**3.8**
	Total Cells Infused	5.24E+08	1.71E+08	1.28E+09	1.07E+09	2.74E+08	5.67E+08	**6.48E+08**
	Body Weight (Kg)	67.3	68.2	54.4	76.8	79.5	68.2	**69.1**
	Cells infused/Kg	7.79E+06	2.51E+06	2.35E+07	1.40E+07	3.45E+06	8.31E+06	**9.93E+06**
	Final CD34%	63.0%	34.0%	12.0%	43.9%	44.3%	80.4%	**46.3%**
	CD34 Cell Expansion (Fold)	4.31	0.81	0.31	1.24	1.86	3.94	**2.08**
	CD34 cells infused/Kg	4.91E+06	8.52E+05	2.82E+06	6.13E+06	1.53E+06	6.68E+06	**3.82E+06**
	Final CD34/41%	30.0%	16.0%	6.0%	18.2%	16.7%	61.0%	**24.7%**
	CD34/41 cells obtained per seeded CD34 cell	2.11	0.38	0.15	0.51	0.70	2.99	**1.14**
	CD34/41 Cells Infused/Kg	2.34E+06	4.01E+05	1.41E+06	2.54E+06	5.76E+05	5.07E+06	**2.06E+06**
	Final CD41%	35.2%	32.0%	27.0%	33.5%	44.6%	74.8%	**41.2%**
	CD41 cells obtained per seeded CD34 cell	2.41	0.77	0.69	0.94	1.87	3.67	**1.73**
	CD41 cells infused/Kg	2.74E+06	8.02E+05	6.35E+06	4.68E+06	1.54E+06	6.22E+06	**3.72E+06**
Release Criteria Results	Infused Cell Viability	70%	70%	**58%**	79%	78%	80%	**73%**
	Bacterial Contamination	**Neg**	ND	ND	**Neg**	**Neg**	**Neg**	
	Fungal Contamination	**Neg**	ND	ND	**Neg**	**Neg**	**Neg**	
	Gram Stain	**Neg**	ND	ND	**Neg**	**Neg**	**Neg**	
	Staph Endotoxin	**Neg**	ND	**Neg**	**Neg**	**Neg**	**Pos**	
	Final Release Status	Infused	NOT infused	NOT infused	Infused	Infused	NOT infused	**3/6**
	Mycoplasma	**Neg**	ND	ND	**Neg**	**Neg**	ND	
	Malignant Contamination	**Neg**	ND	**Neg**	**Neg**	**Neg**	ND	
	Time to 20k Plts (days)	7			12	13		**11**
	Final Platelet Function	Normal	ND	ND	Normal	Normal	ND	
	Anti-TPO Antibodies	ND	ND	ND	ND	ND	ND	
	Residual TPO (ng/ml)	1.2	ND	0	1.8	0.3	0.8	**0.82**

ND = Not determined

4. Role of murine prolactin-like protein E on enhancing the expansion of human megakaryocytes in vitro.[7, Appendix 3]

PLP-E alone did not promote human MK differentiation, but instead synergized with TPO to increase colony-forming unit megakaryocyte (CFU-MK), burst-forming unit erythroid (BFU-E), and colony-forming unit granulocyte erythroid macrophage mixed (CFU-GEMM) expansion, as well as total MK production. These effects can be attributed to an increase in colony frequency combined with a significantly greater total cell expansion enduced by adding PLP-E along with TPO. PLP-E significantly expanded immature, intermediate, and mature CFU-MK subsets at 3 days of culture, as well as the intermediate and mature subsets at day 6. PLP-E combined with TPO induced significant expansion of all CFU-MK subsets at all time points. PLP-E further increased the effect of SCF and Flt-3L on TPO-induced total cell and CFU-MK expansion. In conclusion, PLP-E may act as a survival factor for primitive human megakaryocytic and erythroid progenitors.

5. Molecular mechanism of proplatelet formation.[8, Appendix 4]

Proplatelet formations are precursors for platelet production (PPF). The megakaryoblastic CHRF-288 cell line was used to investigate the mechanism of PPF. The role of fibronectin (FN) and protein kinase C (PKC) were examined. In the presence of serum and phorbol 12-myristate 13-acetate (PMA), a PKC activator, cells exhibited full MK differentiation, manifested by adhesion, shape change, increased cell size, polyploidy, PPF, and expression of CD41+, CD61+ and CD62P+. The same morphologic and phenotypic features were observed in serum-free cultures in the presence of FN/PMA. FN alone induced minimal cell adhesion and spreading, while PMA alone induced only polyploidy without adhesion. Signal transduction changes involved the activation of extracellular signal-regulated protein kinase 1 (ERK1)/ERK2 as well as c-Jun amino-terminal kinase 1 (JNK1)/stress-activated protein kinase (SAPK). Phosphoinositide-3 kinase and p38 were not stimulated under these conditions. Inhibitors were used to identify the causal relationship between signaling pathways and PPF. PD98059 and GF109203X, inhibitors of ERK1/ERK2 pathway and PKC, respectively, blocked PPF, while adhesion, spreading and polyploidy were normal. These studies show that activation of ERK1/ERK2 mitogen-activated protein kinase pathway plays a critical role in PPF. The elucidation of the signal transduction pathway on megakaryocyte development and PPF is of crucial importance for understanding this unique biological process.

KEY RESEARCH ACCOMPLISHMENTS:

- The cocktail of growth factors (thrombopoietin/IL-3/Flt3-L) that optimally stimulates MK ex vivo expansion was identified.
- High density of cell seeding ($4x10^5$ cells/ml) yields higher expansion of MK as compared to low density ($1x10^5$ cells/ml) in both small-scale and large-scale cultures.
- Approval of IND (BB-IND 9559) by the FDA
- Successful platelet engraftment in three patients transplanted with ex vivo expanded MK.
- The Prolactin-Like Protein E (PLP-E) mediates human myeloid precursor survival and expansion. We have added a new effective product to the arsenal of cytokines to

be used in future *ex vivo* expansion protocols involving all human myeloid precursors, including MK.

9 Activation of ERK1/ERK2 mitogen-activated protein kinase pathway plays a critical role in proplatelet, formation which precedes platelet production.

Due to the pitfalls described in the introduction which were beyond our control, we were not able to transplant the number of patients mentioned in the Statement of Work. However, this study showed that using an *ex vivo* expanded product is feasible, and the successful outcome in our three transplanted patients augurs well for future use of this concept.

Although the last two points were not proposed in the original grant, since several months elapsed due to the withdrawal by pharmaceutical companies of various cytokines used in our project and the revision and approval time of revised protocols, we enriched the project with the data obtained, published in well-refereed journals.

REPORTABLE OUTCOMES:

Published Papers (attached in Appendix):

1. Lefebvre, P., Winter, J.N., Kahn, L.E., Giri, J.G., Cohen, I. Megakaryocyte ex vivo expansion potential of three hematopoietic sources in serum and serum-free medium. J. Hematotherapy, 8:199-208 (1999).

2. Lefebvre, P., Winter, J.N., Meng, Y., Cohen, I. Ex vivo expansion of early and late megakaryocyte progenitors. J. Hematotherapy and Stem Cell Research, 9:913-921 (2000)

3. Lefebvre, P., Lin, J., Linzer, D.I.H., Cohen, I. Murine prolactin-like protein E synergizes with human thrombopoietin to stimulate expansion of human megakaryocytes and their precursors. Exp. Hematology, 29:51-58 (2001)

4. Jiang, F., Jia, Y., Cohen, I. Fibronectin- and protein kinase C-mediated activation of ERK/MAPK are essential for proplateletlike formation. Blood, 99:3579-3584 (2002)

5. P Lefebvre and I Cohen. "Large Scale *Ex Vivo* Expansion of Human Megakaryocytes for Clinical Use." in Cytokines and Colony Stimulating Factors: Methods and Protocols Edited by D Körholz and W Kiess. Humana Press, Totowa NJ, 2003.

Paper in preparation to be submitted to Cytotherapy:

Lefebvre, P., Winter, J.N., Cohen, I. Scale up and transplant of ex vivo expanded megakaryocyte progenitors using rhTPO, IL-3 and Flt-3L

Abstracts at Scientific Meetings:

1. Lefebvre, P., Meng, Y.Rl, Miller, W.M., Rademaker, A., Papoutsakis, T., Minster, N., Baum, C.M., McKearn, J.P., Winter, J.N., Cohen, I. Increased megakaryocyte expansion with promegapoietin-progenipoietin combination and high seeding density. ASH meeting, Miami, 1998. Blood 92, Suppl. 1, 1998 (Abstract 2671)

2. Lefebvre, P., Lin, J., Linzer, D.I.H., Cohen, I. A novel murine pregnancy-specific hormone acs as a multilineage survival factor for human bone marrow progenitors. ASH meeting, New Orleans, 1999. Blood 94, Suppl,1, 1999 (Abstract 837)

3. Lefebvre, P., Winter, J.N., Meng, Y., Cohen, I. Expansion of early and late megakaryocyte progenitors using MGDF, SCF and G CSF in a defined-serum medium. ASH meeting, New Orleans, 1999. Blood 94, Suppl, 1999 (Abstract 4783)

4. Jiang, F., Cohen, I. Fibronectin- and protein kinase C-mediated activation of p42/44 mitogen-activated protein kinase are essential for proplatelet formation. San Francisco, 2000. Blood, Suppl. 2000 (Abstract 328).

5. Cohen, I., Jiang, F. Fibronectin- and protein kinase C-mediated activation of ERK/MAPK are essential for proplateletlike formation. XVIIIth Cong. of the Int. Soc. on Thrombosis and Haemostasis, Paris, 2001

CONCLUSIONS:

The results from the three transplanted patients were positive and encouraging. Cell expansion was optimal after 6 days of culture, no negative reactions were observed, and the patients recovered normal platelet levels fast, with normal platelet function. For reasons beyond our control we were not able to transplant the number of patients cited in the Statement of Work, however, this study shows that using an ex vivo expanded product is feasible and the successful outcome in our three transplanted patients augurs well for future use of this concept.

Additionally, we have added a novel survival factor, PLP-E, to the arsenal of cytokines used for *ex vivo* myeloid expansion, including megakaryocytes, and have elucidated a signaling mechanism critical for proplatelet formation.

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9. Lefebvre, P., et al., *Megakaryocyte ex vivo expansion potential of three hematopoietic sources in serum and serum-free medium.* J Hematother, 1999. 8(2): p. 199-208.

Appendix I

JOURNAL OF HEMATOTHERAPY 8:199–208 (1999)
Mary Ann Liebert, Inc.

Megakaryocyte Ex Vivo Expansion Potential of Three Hematopoietic Sources in Serum and Serum-Free Medium

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ABSTRACT

Megakaryocytes (MK) were expanded from purified human CD34$^+$ cells obtained from three sources, bone marrow (BM), mobilized peripheral blood progenitor cells (PB), and umbilical cord (UC) blood. CD34$^+$-selected cells were cultured for 12 days with 10 ng/ml thrombopoietin (TPO), 10 ng/ml IL-3, 10 ng/ml TPO + 10 ng/ml IL-3, or 200 ng/ml promegapoietin (PMP), a chimeric dual agonist of the c-Mpl and human IL-3 receptors. MK production was compared in serum-free versus human serum-supplemented liquid media. PMP and the combination of TPO and IL-3 (TPO + IL-3) increased MK production similarly. Culturing CD34$^+$ cells with PMP in serum-free medium resulted in a twofold increase in MK yield compared with serum-supplemented medium. CD34$^+$ cells from UC proliferated more than those from either BM or PB in liquid culture, resulting in much greater MK production under all conditions. Phenotypic analysis of the uncultured CD34$^+$ cells showed that BM had a higher frequency of CD34$^+$/CD41$^+$ cells than PB or UC. TPO + IL-3 or PMP produced larger and greater numbers of BFU-MK and CFU-MK per seeded CD34$^+$/CD41$^+$ cell from UC than from either BM or PB. Thus, although uncultured CD34$^+$-selected BM cells contained a higher frequency of committed mature MK progenitors, UC CD34$^+$ cells had a greater proliferative capacity and, therefore, were more productive. PMP induced megakaryocytopoietic activity comparable to that achieved with TPO + IL-3 and may be useful for ex vivo expansion of MK for clinical trials.

INTRODUCTION

HEMATOPOIETIC PROGENITOR CELL TRANSPLANTS in which bone marrow (BM), mobilized peripheral blood progenitor cells (PB), or umbilical cord (UC) blood is used to reconstitute hematopoiesis following high-dose chemotherapy is associated with a requisite period of profound thrombocytopenia (1–4). One theoretic approach to shortening the period of thrombocytopenia in patients undergoing autologous PB transplantation is to increase the mobilization of megakaryocyte (MK) progenitors using specific cytokines. The cloning of thrombopoietin (TPO) and its reported activity on all phases of megakaryocytopoiesis and thrombopoiesis (5–11) have raised the possibility of its clinical use for this purpose. However, in view of the restricted pool of stem cells in patients who have received prior chemotherapy, it is often difficult to mobilize adequate numbers of progenitors with the use of cytokines. Experimental studies in rhesus monkeys have shown that TPO administration is ineffective when TPO target cells are reduced by high-dose irradiation (12). Another alternative approach to shortening the period of thrombocytopenia is to administer TPO after transplant to thrombocytopenic patients, although this strategy may be ineffective in raising platelet counts in view of the high levels of endogenous TPO already cir-

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culating in the PB of these patients (13). Supplementing conventional transplants with an MK-rich product may provide an alternate approach to enhancing in vivo platelet production and shortening the period of thrombocytopenia. This was proven effective in one animal study using mice (14). In a clinical trial in which 10 patients received ex vivo expanded MK, 2 of the 4 patients receiving the highest numbers of MK did not require platelet transfusions (15).

TPO has been used in combination with a variety of cytokines to increase the yield of MK in liquid cultures (16–20). Human BM CD34 cells cultured in the presence of TPO and IL-3 produced over threefold more MK than with TPO alone (18). However, IL-3 is not available for clinical use in the United States. Thus, we investigated the megakaryocytopoietic effect of promegapoietin (PMP, G.D. Searle Co., St. Louis, MO), a chimeric dual agonist of both the c-Mpl and human IL-3 receptors (21), to see if it could generate sufficient numbers of MK in vitro for use as a supplement to conventional stem cell autografts. It was reported previously that PMP is superior to TPO or IL-3 alone and comparable to TPO + IL-3 in stimulating in vitro megakaryocytopoiesis in human BM (22). Moreover, experimental studies in nonhuman primates showed that a single dose of PMP hastens the time of platelet recovery (23). In this study, the megakaryocytopoietic capacity of PMP is compared with TPO ± IL-3 using $CD34^+$-selected cells from human BM, UC blood, and PB. Cells were cultured in a serum-containing medium and compared with serum-free medium for the ex vivo expansion of MK.

MATERIALS AND METHODS

Preparation of low-density nonadherent mononuclear cells (MNC)

All samples were collected within the guidelines of the Northwestern University Institutional Review Board on Human Subjects. BM, obtained from the femur of hematologically normal patients having total hip arthroplasty, was collected in anticoagulant designed to prevent platelet activation and containing final concentrations of 50 U/ml preservative-free heparin (GIBCO/Life Technologies, Gaithersberg, MD), 1 mM Na_2 EDTA, 1 mM adenosine, 2 mM theophylline, 2.2 μM prostaglandin E_1 (PGE_1) (Sigma Co., St. Louis, MO), and 0.1 mg/ml DNase I (Boehringer Mannheim, Indianapolis, IN) in 20 ml alpha-modified Eagle's medium (α-MEM, Sigma). BM cells were repeatedly extracted from bone fragments with α-MEM containing 100 U/ml penicillin, 100 μg/ml streptomycin (Boehringer Mannheim), 0.1 mg/ml DNase I (Boehringer Mannheim), and 4 μg/ml gentamicin (Life Technologies). The extract was homogenized by passage through a 21-gauge needle to remove bone fragments. UC blood samples were collected after cesarean section from the freshly delivered placentas of full-term, healthy newborn infants into a 50 ml tube containing 5 ml of anticoagulant without DNase. The blood was diluted 1:1 with α-MEM. BM and UC low-density MNC were isolated with the use of Ficoll-Paque (Pharmacia Corp., Piscataway, NJ) as described (24). PB was collected by continuous flow apheresis from cancer patients destined for high-dose chemotherapy and autologous transplant, following mobilization with G-CSF. PB was cryopreserved in FBS (GIBCO) with 10% DMSO and stored at −120°C until needed (up to 3 months) (25). Ampules were thawed rapidly in a 37°C waterbath and transferred to a thawing medium of α-thioglycerol-free IMDM (GIBCO) containing 20% FBS, 12.5 μg/ml DNase I, 12.5 U/ml heparin, 100 μM Dulbecco's modified Eagle's nonessential amino acids (NEA, GIBCO), 100 U/ml penicillin, and 100 μg/ml streptomycin at 4°C and kept on ice for 15–30 min, then centrifuged at 260*g* for 10 min. Cells were resuspended in α-MEM containing 1% human serum albumin (HSA) (Baxter Hyland, Deerfield, IL) and centrifuged at 380*g* through a 10% HSA cushion in PBS to reduce platelet contamination. Residual red cells were lysed with NH_4Cl (Sigma) as described (26), and the remaining cells were recovered by centrifugation for 6 min at 420*g* through a 10% HSA cushion. Adherent cells from BM, UC, and PB were discarded after overnight incubation at 37°C in a 5% CO_2, fully humidified atmosphere in IMDM containing 10% FBS supplemented with 100 μM NEA, 4 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Purification of $CD34^+$ cells

$CD34^+$ cells were purified using magnetic cell sorting (27) (Mini-MACS kit, Miltenyi Biotec, Auburn, CA) following the manufacturer's recommendations. Cells were passed over two columns and eluted with IMDM. Over 90% of cells were viable with the use of the trypan blue exclusion test, and average purity was 85.2% ± 3.7% as assessed by flow cytometry.

Culture conditions

Cultures from three donors each of BM, PB, and UC were performed. For each cytokine combination, 5×10^4 $CD34^+$ cells were cultured in 1 ml of either IMDM containing 1% HSA and 2.5% pooled normal human serum or commercially available serum-free medium (Easymega, Hemeris, Sassenage, France). Human serum was obtained by recalcification of citrated platelet-free plasma (18) to prevent the inhibitory effects on MK growth of transforming growth factor-β (TGF-β), β-thromboglobulin, and platelet factor 4 released from ac-

tivated platelets (28–30). Cultures were supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and gentamicin (Life Technologies) and maintained for 12 days at 37°C in a 5% CO_2 fully humidified atmosphere. TPO (Zymogenetics Corp., Seattle, WA) (31) was used at a concentration of 10 ng/ml, which yielded the maximal concentration of MK. IL-3 (R&D Systems, Minneapolis, MN) was used at 10 ng/ml. PMP was used at 200 ng/ml. These concentrations were found optimal in earlier dose-response assays (data not shown). One volume of complete culture medium was added at day 6 if excessive cell growth ($>5 \times 10^5$/ml) was observed. After 12 days, cultures were collected, and cells were counted in the presence of trypan blue. Only viable cells were counted and used for calculating MK production.

Clonogenic assay

Clonogenic assays using a serum-free collagen assay (32) (Easymega) were performed according to the manufacturer's instructions. The cytokines used were TPO 10 ng/ml or IL-3 10 ng/ml, or both, or PMP 200 ng/ml. CFU-MK were scored 8 days and BFU-MK were scored 14 days after fixation with methanol/acetone (1:1) and immunostaining with a cocktail of monoclonal anti-GPIb (SZ2-IgG1) and anti-GPIIb (SZ22-IgG1) antibodies (Coulter/Immunotech, Westbrook, ME). Binding of rabbit antimouse IgG was revealed with an APAAP/substrate kit (Dako, Carpinteria, CA).

Flow cytometric analysis

For determination of $CD34^+$ cell purity, up to 2.5×10^5 CD34 cells were treated with 200 pkat chymopapain (Knoll Pharmaceutical Co., Lincolnshire, IL) to detach platelet fragments and eliminate nonspecific GPIIb/IIIa attached to $CD34^+$ cell surfaces (33). For analysis of MK progenitors ($CD34^+/CD41^+$), cell aliquots were washed in phosphate-buffered albumin (sPBA) designed to prevent platelet activation and containing 13.6 mM sodium citrate·$2H_2O$, 11 mM dextrose, 1 mM theophylline, 2.2 μM PGE_1, and 10% BSA (Sigma) in Dulbecco's PBS, pH 7.4. After washing, the cells were stained for 15 min on ice in the dark with PE-conjugated-α-CD34 (HPCA-2, Becton, Dickinson, San Jose, CA) and FITC-α-CD41 (Coulter/Immunotech) and analyzed by flow cytometry. The negative controls were PE- and FITC-α-mouse IgG1 used at equivalent IgG1 concentrations. The relative frequency of mature MK was measured after 12 days in culture by flow cytometric analysis of cells stained with FITC-α-CD41a. Cells were washed again in sPBA and fixed with 1% paraformaldehyde (Ted Pella, Redding, CA) containing 0.067 M sodium cacodylate (Sigma) in saline. Flow cytometric analysis was performed using a Coulter Cytometry XL (Coulter Co., Hialeah, FL) flow cytometer. Fluorescence attributable to FITC-labeled and PE-labeled antibodies was determined using excitation by an argon laser operating at 488 nm. Emissions from FITC and PE were measured using band pass filters of 525 nm and 585 nm, respectively. Approximately 10,000 events were counted per acquisition. The integration region for the $CD34^+$ cells was set around the uppermost positive population of cells. The integration region for the $CD41^+$ cells was set at 1% of the negative population. The percent positive cells was calculated by subtracting the percent positive of the isotype control within the same integration region.

RESULTS

Evaluation of culture media

BM $CD34^+$ cells were cultured with 200 ng/ml PMP in serum-free medium, and total production of MK was compared with that obtained by culturing cells from the same donor in IMDM with 2.5% human serum (Fig. 1). Compared with human serum-containing medium, incubation in serum-free medium resulted in increased MK frequency and cell proliferation.

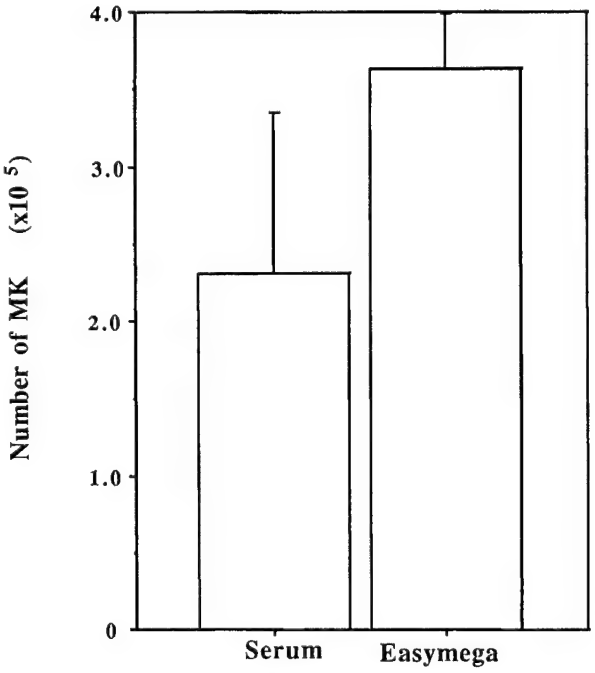

FIG. 1. MK productivity in the presence of serum-free medium. MK productivity was calculated on the basis of the relative frequency of MK and the total number of trypan blue-negative cells. Using PMP, serum-free medium (Easymega) produced more MK than did serum-containing medium.

Total cell proliferation

UC-derived $CD34^+$ cells had higher total cell proliferation than either BM or PB under all conditions, whereas there were no differences between BM and PB (Fig. 2). In serum-containing medium, IL-3 induced greater cell proliferation than TPO for BM and PB but not UC, and combining TPO with IL-3 led to a cooperative increase in cell proliferation compared with TPO alone and IL-3 alone (Fig. 2A). In the same culture conditions containing UC cells, the proliferative effect of PMP or TPO + IL-3 was additive compared with TPO or IL-3 alone. For UC, TPO + IL-3 induced greater cell proliferation than PMP in the presence of serum (Fig. 2A), whereas the inverse was true in serum-free medium (Fig. 2B).

Relative frequency of ex vivo expanded megakaryocytes

For each hematopoietic source, the percentage of $CD41^+$ cells was greater in PMP, TPO, and TPO + IL-

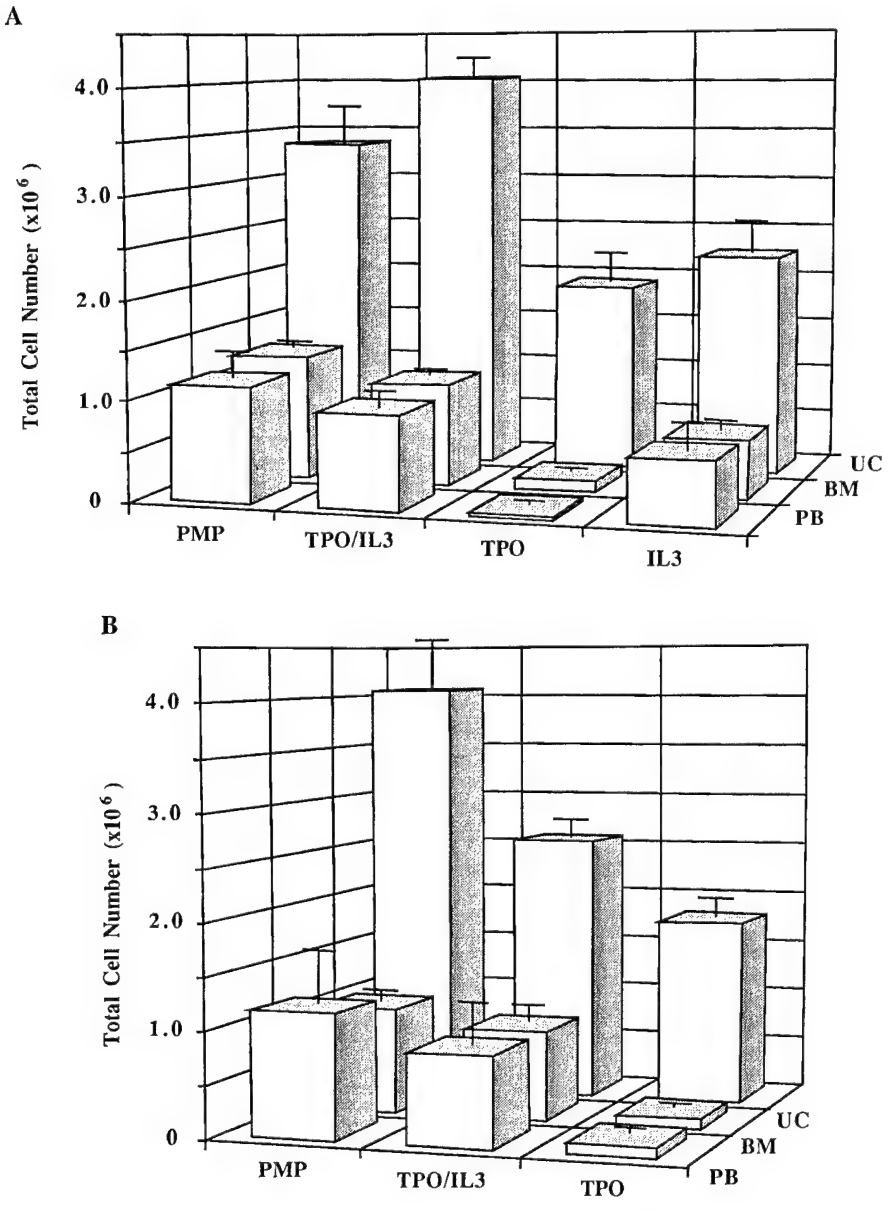

FIG. 2. Total cell proliferation. The number of trypan blue-negative cells per milliliter of culture was counted for each sample. The mean ± SEM total cell proliferation is shown. **(A)** Cells cultured in serum-containing medium. **(B)** Cells cultured in serum-free medium.

3-supplemented cultures when compared with IL-3-supplemented cultures in serum-containing medium (Fig. 3A). In these culture conditions, PMP induced a lower $CD41^+$ cell frequency than did TPO alone (Fig. 3A). When BM cells were cultured in serum-free medium, TPO induced lower $CD41^+$ cell frequencies than either TPO + IL-3 or PMP (Fig. 3B). Cultured BM and UC cells exhibited higher $CD41^+$ cell frequencies than PB for all cytokine and culture medium combinations.

Total MK production

Overall, using either TPO + IL-3 or PMP resulted in greater MK production compared with TPO or IL-3 alone (Fig. 4). The addition of IL-3 to TPO cooperatively increased MK production for BM and PB compared with using TPO or IL-3 alone (Fig. 4A). The same cooperativity was seen with PMP. For UC in the presence of serum, the effect of TPO + IL-3 or PMP was additive

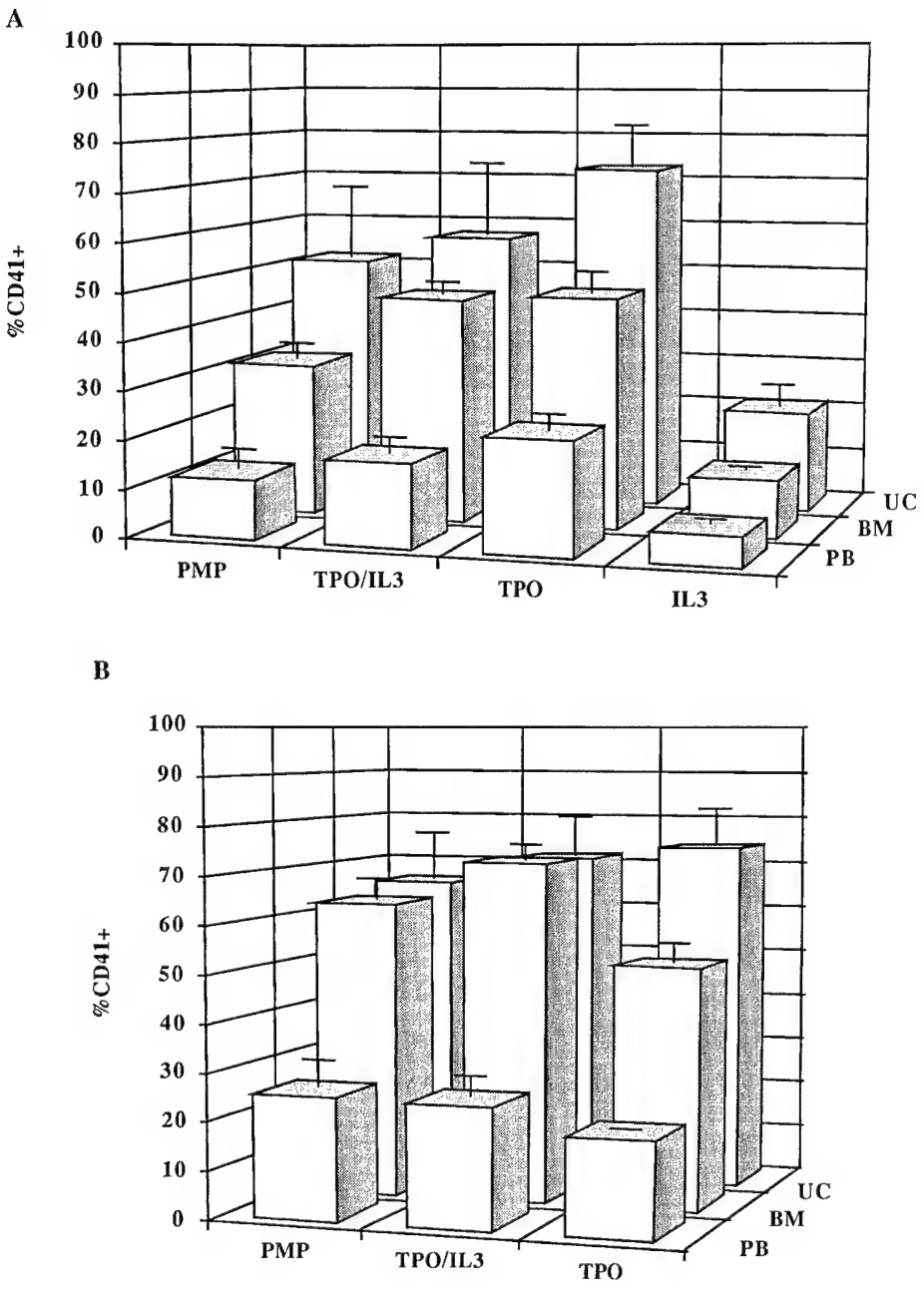

FIG. 3. Frequency of MK in culture. The mean ± SEM $\%CD41^+$ for each sample is shown, as measured by flow cytometry. **(A)** Cells cultured in serum-containing medium. **(B)** Cells cultured in serum-free medium.

compared with TPO or IL-3 alone. UC cultures produced greater numbers of MK than BM or PB under all conditions (Fig. 4), and BM produced more MK than PB. Higher numbers of UC MK were obtained with TPO + IL-3 when compared with PMP in the presence of serum, and the inverse occurred in serum-free medium.

Clonogenic capacity of CD34$^+$ cells

There were no differences between TPO + IL-3 and PMP in the number of either CFU-MK or BFU-MK colonies produced from any cell source (Table 1). Samples cultured with either TPO + IL-3 or PMP showed an

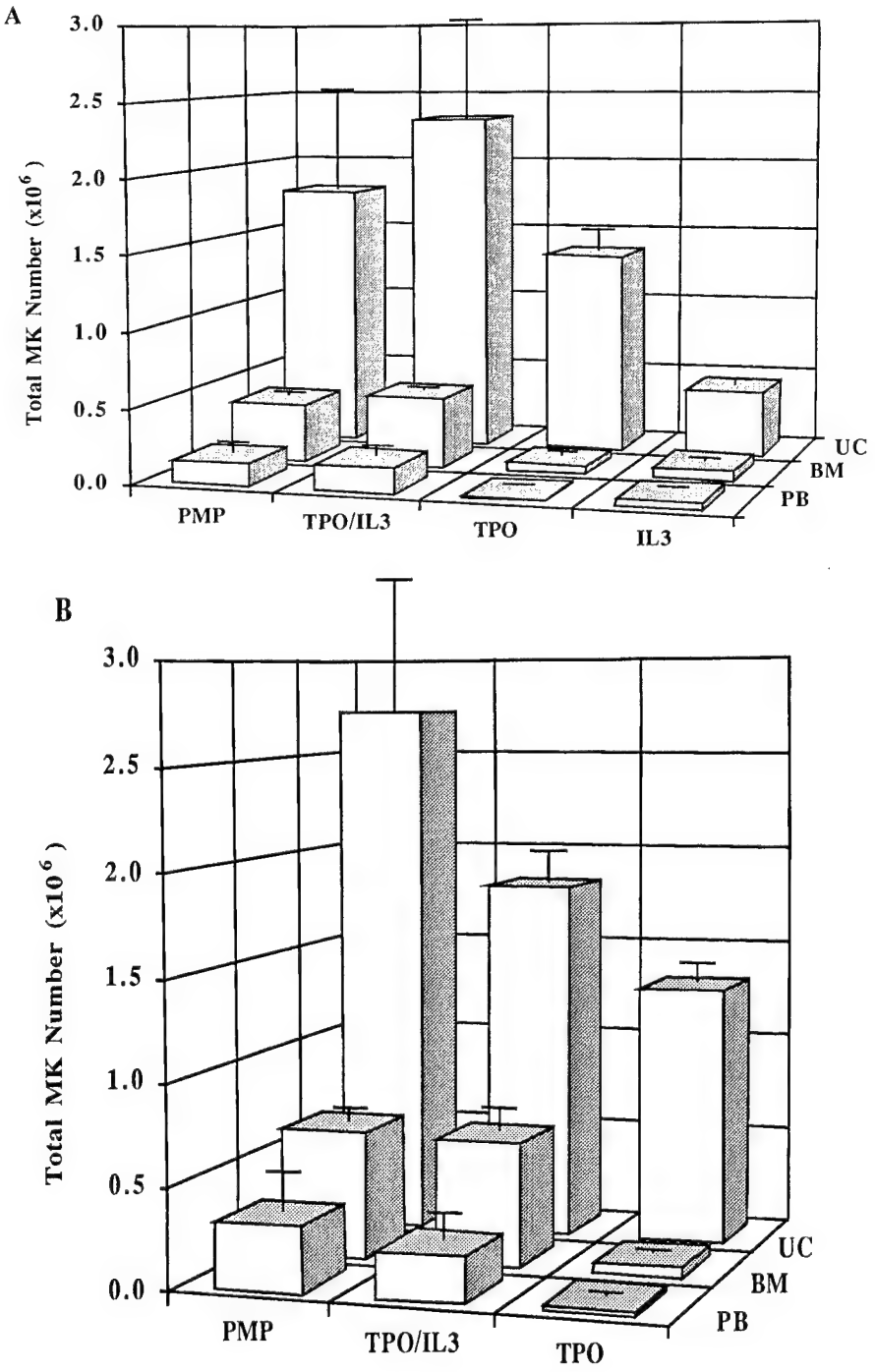

FIG. 4. Total number of MK produced for each condition. The mean ± SEM number of MK, calculated by multiplying the total cell number by the MK frequency, for each sample is shown. **(A)** Cells cultured in serum-containing medium. **(B)** Cells cultured in serum-free medium.

TABLE 1. CLONOGENIC CAPACITY OF $CD34^+$ CELLS[a]

	CFU-MK		*BFU-MK*	
Cell source	*PMP*	*TPO/IL-3*	*PMP*	*TPO/IL-3*
UC (n = 5)	157 ± 26	149 ± 13	133 ± 39	109 ± 20
BM (n = 5)	84 ± 29	88 ± 23	47 ± 11	39 ± 10
PB (n = 5)	27 ± 4	32 ± 2	12 ± 2	11 ± 1

[a]Total number of colonies measured ± SEM. Each sample was analyzed in duplicate.

additive increase in the number of CFU-MK and BFU-MK when compared with samples exposed to TPO alone or IL-3 alone (data not shown). UC $CD34^+$ cells produced more CFU-MK and BFU-MK colonies than did BM or PB cells (Table 1). The size of BFU-MK from UC (>200 cells per colony) was larger than the equivalent colonies from BM or PB (data not shown). In separate experiments, the frequency of MK progenitors ($CD34^+/CD41^+$) in the initial $CD34^+$ cell population was measured by flow cytometry. $CD34^+/CD41^+$ cell frequency was greater for BM than for the other two hematopoietic sources (Table 2). There was no difference between UC and PB $CD34^+/CD41^+$ cell frequency. When the ratio of CFU-MK or BFU-MK colonies per seeded $CD34^+/CD41^+$ cells was calculated, UC cells produced a higher ratio than either BM or PB (Table 2).

DISCUSSION

In this study, we investigated whether PMP, a novel chimeric cytokine, could substitute for the combination of TPO and IL-3 in ex vivo expansion of MK and provide sufficient numbers for use as a supplement to stem cell transplants. PMP had comparable in vitro biologic activity to TPO + IL-3, and the serum-free medium we tested was superior to human serum-containing medium in supporting megakaryocytopoiesis.

Both PMP and TPO + IL-3 cooperatively increased MK production compared with either TPO or IL-3 alone in both BM and PB liquid cultures. The data show that TPO exerts its megakaryocytopoietic effect by increasing the frequency of $CD41^+$ cells in culture, whereas IL-3 increases total cell proliferation, particularly in BM and PB. In the more proliferative UC $CD34^+$ cells, the effects of TPO and IL-3 were nearly equivalent. IL-3 is known to stimulate megakaryocytopoiesis independently of TPO (34,35), and combining TPO and IL-3 cooperatively increases megakaryocytopoiesis, as previously shown (16–19). This cooperativity may be because TPO and IL-3 can transmit some of their mitogenic signal through different cell signaling pathways (36–38) and amplify each other's signals. The design of PMP, a chimeric protein that activates both the c-Mpl and IL-3 receptors, can take advantage of these overlapping signal transduction pathways (39,40).

Depending on the cytokine combination used, the composition of the culture medium greatly affects MK production. In this respect, UC cells were more sensitive to PMP in serum-free than in serum-containing medium. The inverse was true for the combination of TPO and IL-3. The specific effect of components of culture medium, such as cannabinoid ligands, present in serum was reported recently (41). It was surmised that they affect cell production by transactivation of hematopoietic receptors, potentiation of signal transduction mediated by hematopoietic receptors, or activation of signalling pathways that enhance cell cycling in parallel with hematopoietic receptor transduction pathways. Moreover, in view of the different topography of their receptors, different

TABLE 2. MK COLONIES DERIVED FROM MK PROGENITORS[a]

Cell source	*%$CD34^+/CD41^+$ ±SEM (n)*	*CFU-MK per $CD34^+/CD41^+$ cell ±SEM (n)*	*BFU-MK per $CD34^+/CD41^+$ cell ±SEM (n)*
UC	1.8% ± 1.1% (22)	4.5 ± 0.7 (18)	3.1 ± 0.4 (18)
BM	4.0% ± 1.8% (19)	1.0 ± 0.1 (26)	0.4 ± 0.1 (21)
PB	1.6% ± 1.5% (23)	1.6 ± 0.3 (20)	0.7 ± 0.2 (12)

[a]Results are for colonies cultured with TPO + IL-3. % $CD34^+/CD41^+$ determined by flow cytometry. CFU-MK and BFU-MK per $CD34^+/CD41^+$ cell was calculated by dividing the number of colonies by the number of $CD34^+/CD41^+$ cells seeded.

hematopoietic sources may not be equivalent in signalling pathways. These considerations may explain the different responses of cytokine combinations depending on the culture medium and the hematopoietic source used.

The increased frequency and production of $CD41^+$ cells in BM as compared with PB were proportional to the $CD34^+/CD41^+$ frequency in the initially seeded population. Although UC cells had fewer $CD34^+/CD41^+$ cells, they were more proliferative than BM or PB cells. This led to greater MK production in UC cultures compared with PB or BM cultures. This may be because UC has a higher frequency of highly proliferative primitive progenitors (42,43). Results of clonogenic assays showed that UC produced more CFU-MK and BFU-MK colonies than the other two hematopoietic sources. In addition, these colonies were much larger in cell number, and the size and number of colonies were proportional to the proliferative potential of the cells, as previously reported (44). Interestingly, whereas BM and PB produced about as many colonies as there were MK progenitors ($CD34^+/CD41^+$ cells) in the initial seeded cell population, UC cells produced 4.5-fold and 3-fold more CFU-MK and BFU-MK, respectively, than the initial number of seeded MK progenitors would predict. This suggests that the more primitive UC $CD34^+$ cells may be more easily recruited to the MK lineage by a c-Mpl ligand and would, therefore, generate more MK in culture.

Using a cocktail of seven cytokines, including MIP-1α, IL-3, IL-6, IL-11, Flt-3L, SCF, and MGDF, in their ex vivo expansion protocol, Bertolini et al. (15) transplanted an enriched MK product into patients (15). Two of the four patients who received the highest MK infusions ($2.5–21.3 \times 10^5$ $CD61^+$ cells/kg) did not require platelet transfusions. Our goal is to transplant a minimum of 21.3×10^5 MK/kg in the patient. Whereas Bertolini et al. (15) were able to harvest an average of 4.1 MK per cell seeded, we have shown that PB $CD34^+$ cells cultured in serum-free medium with a single chimeric cytokine, PMP, will generate an average of 6.4 MK per cell seeded. Based on these figures, for a 70 kg patient, 2.3×10^7 $CD34^+$ cells should be cultured for 12 days to reach our goal, well within technical and clinical limits. BM cells were twice as productive and UC cells 8.5-fold more productive as PB, further reducing the starting material required.

In conclusion, it is feasible to expand MK from human $CD34^+$ cells ex vivo, producing a cellular product that could have important clinical use. Although UC cells were the most productive hematopoietic source on a per volume basis because of their high concentration of primitive progenitors, the MK yield observed with PB will be more than sufficient for clinical use. These preclinical studies demonstrate that the single chimeric cytokine PMP should yield sufficient numbers of MK for clinical trials, avoiding the difficulties associated with the use of cytokines not approved for clinical use and with multiple corporate sources.

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Appendix II

Ex Vivo Expansion of Early and Late Megakaryocyte Progenitors

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ABSTRACT

Our goal is to produce *ex vivo*-expanded human megakaryocytes (MK) cells from peripheral blood progenitor cell (PBPC) harvests for use in supplementing conventional autografts. In this paper we show the megakaryocytopoietic productivity of small-scale *in vitro* serum-free cultures of human $CD34^+$ cells containing MK growth and development factor (MGDF) and stem cell factor (*Kit* ligand; SCF) ± granulocyte colony-stimulating factor (G-CSF). Cultures were characterized after 3, 6, 9, and 13 days by flow cytometry and clonogenic assays. $CD34^+$ cells expanded 5.2- and 3.4-fold, and produced 2.2 and 2.4 $CD34^+/41^+$ cells per seeded $CD34^+$ cell after 6 and 9 days in culture, respectively. None were detected at day 13. $CD41^+$ cells expanded exponentially over 13 days. Colony-forming unit-megakaryocyte (CFU-MK) also expanded exponentially, but the proportion of the most primitive CFU-MK dropped from 45% to 1.5% and to <1% after 6 and 9 days, respectively. G-CSF increased total cell expansion, but decreased $CD41^+$ frequency, yielding no gain in MK production. We also found that PB $CD34^+$ cells cultured for 3–6 days are richer in primitive MK progenitors, while those cultured for 9–13 days have greater numbers of more differentiated MKs. Overall, the combination of MGDF+SCF proved sufficient for expanding $CD34^+/CD41^+$ cells. As the stage of *ex vivo* MK differentiation most conducive to optimal platelet production *in vivo* is not known, we are planning a clinical trial to determine the efficacy of *ex vivo*-expanded MKs on platelet recovery in relation to MK maturity.

INTRODUCTION

PATIENTS UNDERGOING HPC TRANSPLANTS to reconstitute hematopoiesis following myeloablative chemotherapy usually engraft neutrophils within 1–2 weeks, but often suffer from severe thrombocytopenia for as long as 2–3 weeks (1,2). Unfortunately, platelet transfusions are risky and costly (3), prompting a search for alternatives. Following the discovery of thrombopoietin (TPO) it was hypothesized that its administration, or analogs such as megakaryocyte growth and development factor (MGDF; [4,5]) to post-transplant thrombocytopenic patients might help to shorten the period of post-transplant thrombocytopenia (6). Unfortunately, preliminary results from phase I clinical trials using TPO or MGDF have shown little or no effect on platelet recovery following myeloablative chemotherapy (7–11).

It has been shown that the rate of platelet recovery following stem cell transplantation correlates with the number of infused colony-forming unit-megakaryocyte (CFU-MK) and/or $CD34^+/41^+$ cells (12–14). These cells, which represent the megakaryocyte (MK) progenitor population, most likely promote platelet engraftment. While it is not always possible to obtain a sufficient number of MK progenitors for rapid platelet engraftment from mobilized PBPC collections alone, supplementation of conventional autografts with an *ex vivo*-expanded MK-rich product may enhance *in vivo* platelet production and shorten the period of thrombocytopenia. This approach was shown to be effective in a study in which *ex vivo*-

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[3]Preliminary results of this paper were presented as an abstract at the 1999 American Society of Hematology meeting.

expanded MK progenitors were administered to mice (15).

TPO alone has little proliferative effect on human $CD34^+$ cells in culture. Studies have shown that the addition of early acting cytokines is required to increase total cell proliferation and MK expansion (16–18). This approach has been used in three phase I clinical trials in which the unprimed PBPC autograft was supplemented with an *ex vivo*-expanded MK-rich product, with variable effects on platelet recovery reported. In a clinical trial involving ten patients undergoing stem cell transplant who received cells expanded with MGDF, SCF, IL-3, IL-6, IL-11, Flt-3-ligand, and macrophage inflammatory protein-1α, two out of the four patients receiving the highest numbers of infused MKs did not require platelet transfusions (19). In two other clinical trials using a combination of MGDF, SCF and G-CSF, the results were variable. One study reported sustained engraftment of neutrophils and platelets (20), while the other showed only an effect on neutrophil engraftment (21). In neither study were the expanded MKs characterized in terms of numbers of MK progenitors and/or differentiated MKs. This information would be valuable to correlate platelet response with the quality and quantity of MKs and MK progenitors. In this paper, we have characterized MK development at different durations of culture for the purpose of designing a clinical trial that will determine the ratio of expanded progenitors/differentiated MK most conducive to *in vivo* platelet engraftment.

MATERIALS AND METHODS

Preparation of low density nonadherent mononuclear cells from peripheral blood aphereses

All samples were collected after informed consent was obtained within the guidelines of the Northwestern University Institutional Review Board on Human Subjects. PBMC were collected by continuous flow apheresis from four breast cancer patients eligible for high-dose chemotherapy and autologous transplant and mobilized with G-CSF. Aliquots of PBMC were cryopreserved in fetal bovine serum (FBS) (GIBCO/Life Technologies, Gaithersberg, MD) with 10% DMSO and stored at −120°C until needed (up to 4 months). Ampules were thawed in a 37°C water bath and transferred to a thawing medium of α-thioglycerol-free Iscove's modified Dulbecco's medium (IMDM) (GIBCO) containing 20% FBS, 12.5 μg/ml DNase I (Boehringer Mannheim, Indianapolis, IN), 12.5 U/ml preservative-free heparin (GIBCO), 100 μM Dulbecco's Modified Eagle's nonessential amino acids (NEA, GIBCO), and 100 U/ml penicillin/100 μg/ml streptomycin (PS, Boehringer Mannheim) at 4°C, kept on ice for 15–30 min, then centrifuged at 260 × g for 10 min. Residual RBCs were lysed with Ortho-Mune™ lysing reagent (Ortho Diagnostic Systems, Raritan, NJ) and the remaining cells recovered by centrifugation for 6 min at 420 × g through a 10% human serum albumin (HSA) (Baxter Hyland, Deerfield, IL) cushion. Adherent mononuclear cells were discarded following 2-h incubation at 37°C in a 5% CO_2, fully humidified atmosphere, in IMDM containing 10% FBS supplemented with 100 μM NEA, 4 μg/ml gentamicin, and PS.

Purification of $CD34^+$ cells

$CD34^+$ cells were purified using magnetic cell sorting (22) (MiniMACS System, Miltenyi Biotec, Auburn, CA) following the manufacturer's recommendations. Cells were passed over two columns and eluted with defined serum-free medium (Amgen, Thousand Oaks, CA). An average of 56.3 ± 8.6% of cells were viable as measured by the Trypan blue exclusion test and average purity was 60.7 ± 12.5% as assessed by flow cytometry.

Culture conditions

For each cytokine combination, 4×10^5 cells/ml were cultured in defined serum-free medium (Amgen), with MGDF and SCF (Amgen), with or without G-CSF (Amgen), all at 100 ng/ml. Cultures were maintained for 13 days at 37°C in a 5% CO_2 fully humidified atmosphere. Cultures were resuspended daily, and cells counted in the presence of Trypan blue. If cell concentration was $\geq 8 \times 10^5$, it was readjusted to 4×10^5 by diluting with fresh medium and cytokines. Clonogenic and flow cytometric analyses were performed at days 0, 3, 6, 9, and 13. Only the Trypan blue-negative cells were counted and used for calculating cell proliferation and MK production.

Flow cytometric analysis

Cell aliquots were washed in 1% BSA (Sigma) in PBS (GIBCO) with 5 mM EDTA (Sigma), designed to prevent further platelet activation and/or reverse adherence of activated platelets (13). After washing, the cells were stained for 15 min at 4°C in the dark with phycoerythrin-cyanin 5.1 (PC5) conjugated-α-CD34 (Clone 581, Coulter-Immunotech, Miami, FL) and either a combination of FITC-α-CD41 (Coulter-Immunotech) and PE-conjugated annexin V (R&D Systems, Minneapolis, MN) in the presence of 1% BSA-PBS + 2.5 mM $CaCl_2$, or a combination of FITC-α-CD15 (Coulter-Immunotech) and PE-α-CD11 (Coulter-Immunotech), and analyzed by flow

cytometry. The negative controls were PC5-, PE- and FITC-α-mouse IgG_1 used at equivalent IgG_1 concentrations, and PE-Annexin V without calcium. Only the non-apoptotic high forward-scatter, low side-scatter (predominantly annexin V-negative) cell population was used for subset analysis (see Fig. 2**A**).

DNA degradation products (<2N DNA), reflecting apoptosis, and MK ploidy content were also measured by flow cytometry. CD41/annexin V-stained cells were washed again in 1% BSA-PBS and resuspended in 1 mg/ml sodium citrate with 50 μg/ml 7-aminoactinomycin D (Sigma), then incubated for >30 min at 4–8°C in the dark (23). Samples were not fixed, but analyzed on the same day using three-color laser (Coulter Epics XL, Coulter Corp, Miami, FL).

Clonogenic assay

Clonogenic assays using a serum-free collagen medium containing TPO, IL-3 and IL-6 (MegaCult-C, Stem Cell Technologies, Vancouver, BC) for CFU-MK, and a serum-free methylcellulose medium containing SCF, GM-CSF, IL-3, IL-6 G-CSF, and EPO (MethoCult GF+, Stem Cell Technologies) for CFU-GM and BFU-E were performed according to the manufacturer's instructions. Briefly, viable cells from each day of assay were seeded at 10^3/chamber. CFU-MK were scored after ten days by labelling with mouse anti-human CD41 followed by goat anti-mouse biotin-conjugated IgG. MK colonies were resolved by staining with avidin-conjugated alkaline phosphatase, reacting with substrate, and counter-staining with Evans blue. CFU-MK maturity was evaluated by counting the number of cells in each colony. Three size categories were distinguished: small mature colonies were those with ≤20 cells, medium-size colonies contained 21–50 cells, large immature colonies contained >50 cells. CFU-GM/BFU-E were scored after 14 days. BFU-E colonies were visually distinguishable by light-dense heme expression.

Statistical analysis

Paired comparisons were analyzed using the Wilcoxen signed rank nonparametric test. Statistical significance was given to any result with $p < 0.05$.

RESULTS

Total cell expansion

While there was no significant expansion of total cells at day 3, cell number increased exponentially thereafter (Fig. 1). The addition of G-CSF to MGDF+SCF significantly increased total viable cell proliferation three-fold at day 13, but had no significant effect at earlier time points. Despite the significant increase in total cell number, the frequency of nonviable cells did not significantly change during the culture period (Fig. 2A).

$CD34^+$ and $CD41^+$ cell expansion

Using MGDF+SCF, $CD34^+$ cell frequency increased significantly during the first three days, but then decreased as cells differentiated, until $CD34^+$ cells were not reliably detected at 13 days (Figs. 2**B** and 3**A**). Over time, the proportion of $CD34^+$ cells that were also $CD41^+$ increased (Fig. 3**A**). Due to overall cell proliferation the total number of $CD34^+$ and $CD34^+/41+$ cells per seeded $CD34^+$ cell significantly expanded at 6 and 9 days compared with day 0 (Fig. 3**B**). No differences were seen when G-CSF was added to the cultures (data not shown).

As $CD34^+$ cells matured and became committed to the MK lineage, total $CD41^+$ cell production ($CD34^+/41^+$ plus $CD34^-/CD41^+$) increased exponentially, in parallel with total cell proliferation and the increase in $CD41^+$ frequency (Figs. 3**A** and **B**). $CD41^+$ cell frequency increased with MGDF+SCF with or without G-CSF at day 3, but there was no increase in $CD41^+$ frequency after day 3 in the presence of G-CSF (data not shown). Overall, the increased total cell proliferation induced by G-CSF (Fig. 1) combined with the lower $CD41^+$ frequency led to production of equal numbers of $CD41^+$ cells, whether or not G-CSF was added (data not shown). In the presence of MGDF+SCF, mean frequency of

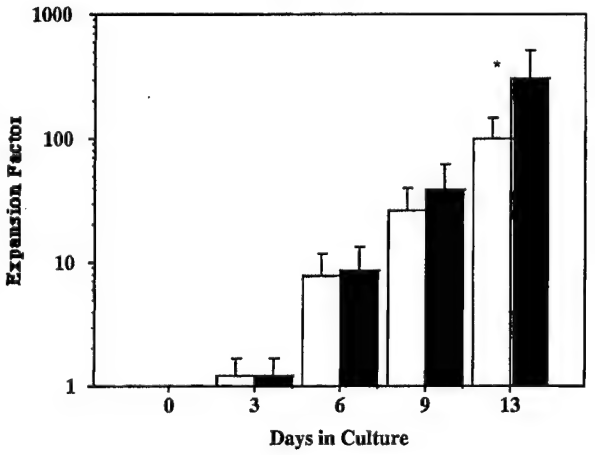

FIG. 1. Total cell expansion. The number of Trypan blue negative cells was counted at each time point, and divided by the number of seeded cells at day 0. □ MGDF+SCF; ■ MGDF+SCF+G-CSF. *Adding G-CSF significantly increased total cell expansion at day 13: $p < 0.05$.

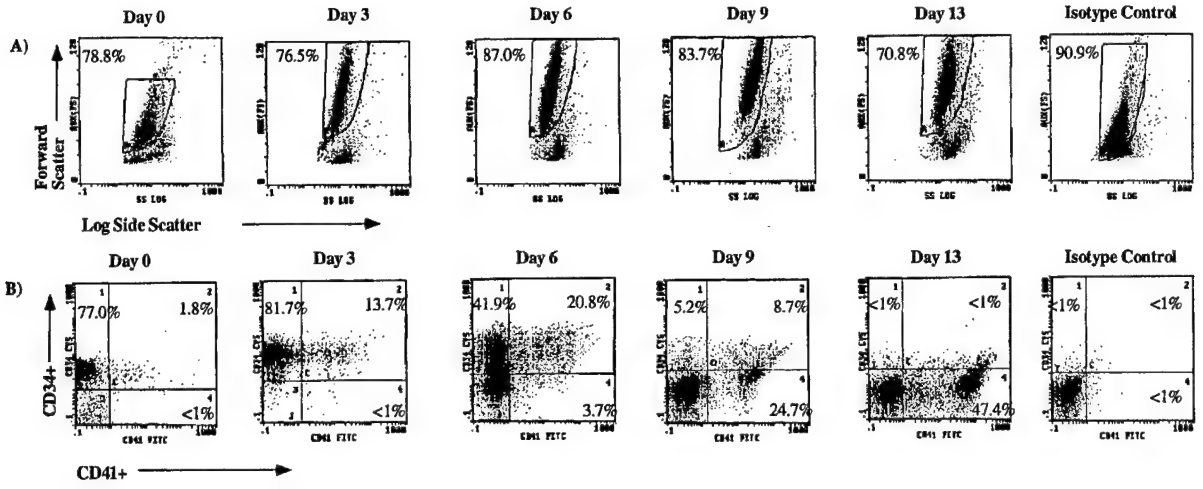

FIG. 2. Changes in cell culture composition. Representative flow cytometry patterns showing the changes in cell viability and megakaryocyte development over 13 days of culture. (**A**) Cell viability: The population of cells demonstrating high forward scatter and low side scatter was considered the viable cell population. The percentage of viable cells is marked in each histogram. Viable cells were >90% annexin V-negative. Nonviable cells were predominantly annexin V-positive, indicating they had begun to apoptose. (**B**) Megakaryocyte development: The viable cell population in (A) was analyzed for CD34+ and CD41+ cell content (as well as CD15+ cell content, not shown). The percentage of $CD34^+/41^-$, $CD34^+/41^+$ and $CD34^-/41^+$ cells for each day is marked in the respective quadrants.

megakaryocytes with a ploidy greater than 4N reached 7.6 ± 3.3% at day 13, with most of these cells being only 8N. When G-CSF was added to MGDF+SCF, the mean frequency of megakaryocytes with a ploidy greater than 4N decreased insignificantly to 6.7 ± 0.8% at day 13.

Colony forming unit cell expansion

CFU-GM (Fig. 4**A**) and BFU-E frequencies (Fig. 4**B**) were proportionally similar to $CD34^+$ frequency (Fig. 3**A**), also peaking at day 3, and declining thereafter. G-CSF treatment had no significant effect on any colony frequency (Fig. 4) or expansion (data not shown). CFU-MK frequencies were an order of magnitude less than but paralleled $CD34^+/41^+$ frequency, except at day 13, where CFU-MK, but not $CD34^+/41^+$ cells (Fig. 3**A**), could be reliably detected in all samples tested (Fig. 4**C**). Total CFU-MK (Fig. 5) increased rapidly in parallel with total cell proliferation and total $CD41^+$ cells (Fig. 3**B**). However, there was a dramatic shift in the proportion of immature versus mature colonies. Total number of immature colonies, defined as CFU-MK with >50 cells per colony, expanded slightly at day 3, but then decreased rapidly by days 6 and 9. The proportion of immature CFU-MK in relation to the total CFU-MK population also decreased dramatically, from nearly 50% at day zero to undetected at day 13, in parallel with $CD34^+$ cell frequency. Total numbers of intermediate-sized colonies (21–50 cells) peaked at day 6, then decreased, while their proportion changed in a manner similar to $CD34^+/CD41^+$ cell frequency. The most mature colonies (≤20 cells/colony) paralleled total $CD41^+$ cells. This correlates with the flow cytometric results, which showed that the vast majority of $CD34^+/41^+$ cells at day 9 were much dimmer in their $CD34^+$ staining than at day 6, indicating a more mature, differentiated population (Fig. 2B, mean $CD34^+$ PC5 fluorescence at day 6 = 8.83, peak = 10.2, Mean PC5 fluorescence at day 9 = 5.34, peak = 3.01).

DISCUSSION

Our goal is to produce a MK-rich product to be used as a supplement to conventional autografts to alleviate post-transplant thrombocytopenia. Our results show that the ratio of early MK progenitors versus differentiated megakaryocytes in the *ex vivo* expanded product changed dramatically over time. Cultures assayed at day 3 showed that, despite little total cell expansion, there was an increase in the number of early progenitors. As the culture progressed, early progenitor frequencies decreased, but their numbers expanded, driven by total cell expansion. Differentiated megakaryocytes emerged as the MK progenitors matured. Therefore, the maturation distribution of MK in a clinical *ex vivo* expansion protocol will be dependent upon the time of cell harvest.

We previously reported that cell seeding density can affect the expansion of MK progenitors (24). While seeding densities of 5×10^4 to 10^5 $CD34^+$ cells/ml were used

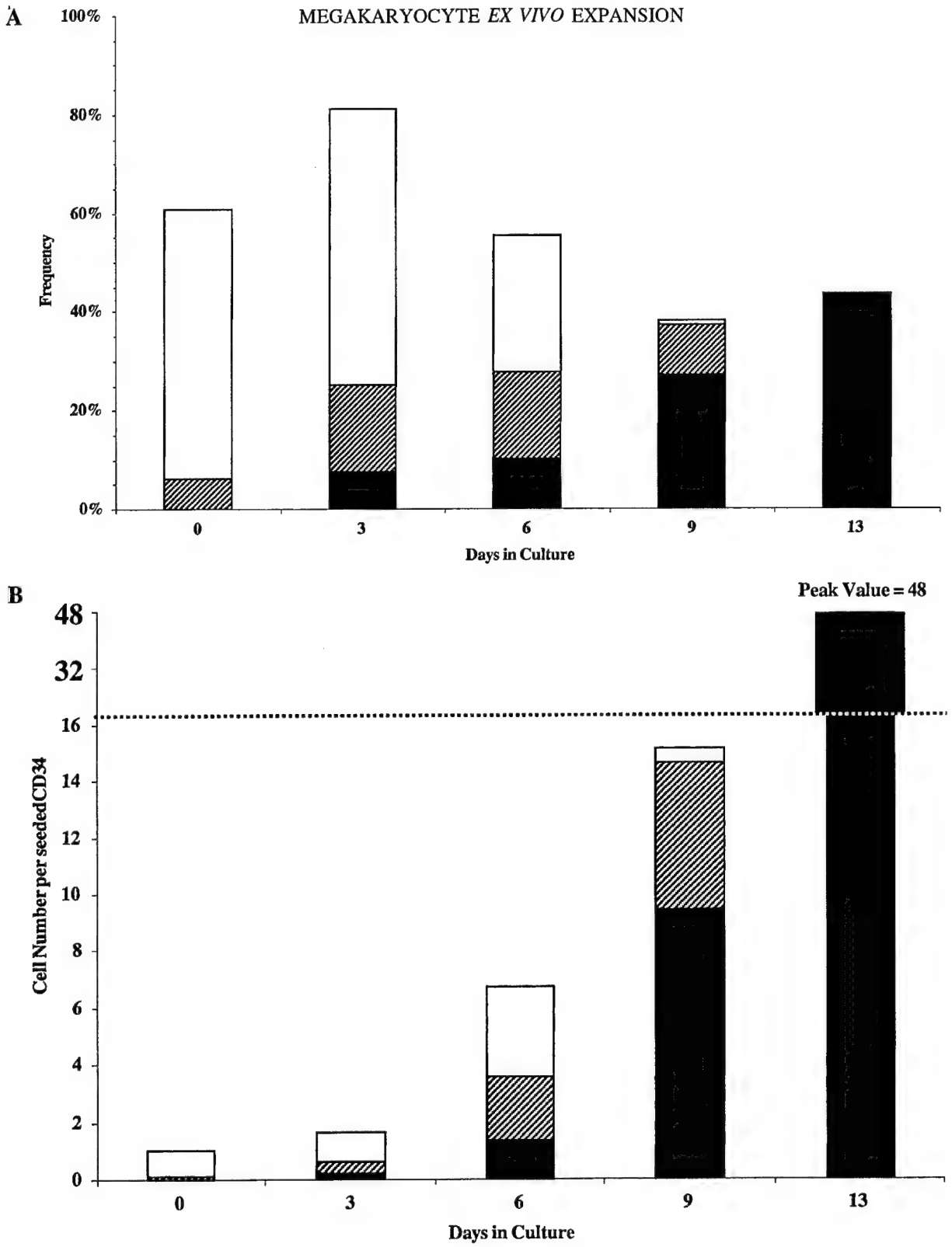

FIG. 3. Target cell expansion. (**A**) $CD34^+/41^-$, $CD34^+/41^+$, and $CD34^-/41^+$ cell frequency. The frequency of $CD34^+$, $CD34^+/41^+$, and $CD41^+$ cells in the viable cell population using MGDF+SCF were measured by flow cytometry. □ $CD34^+/CD41^-$; ▨ $CD34^+/41^+$; ■ $CD34^-/41^+$. (**B**) $CD34^+/41^-$, $CD34^+/41^+$, and $CD34^-/41^+$ cell expansion. The total number of viable $CD34^+$, $CD34^+/41^+$, and $CD41^+$ cells found at each day were divided by the number of $CD34^+$ cells seeded at day 0 to obtain total production per seeded $CD34^+$ cell. □ $CD34^+/CD41^-$; ▨ $CD34^+/41^+$; ■ $CD34^-/41^+$. Notice change of scale in **B**.

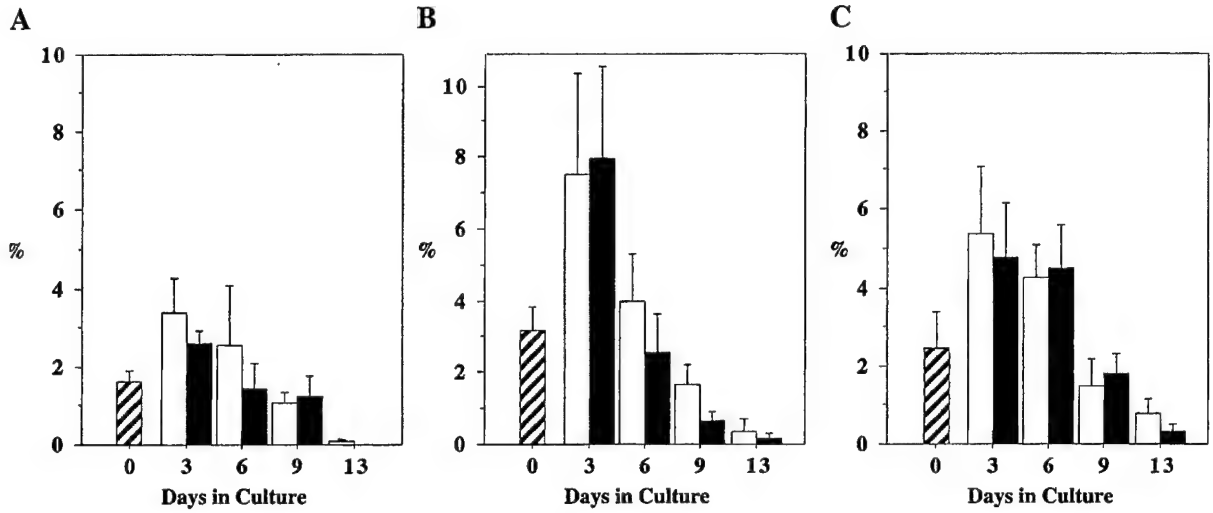

FIG. 4. Colony-forming cell frequency. The percentage of 1000 seeded cells, obtained from liquid cultures after the indicated time, that developed into the various colonies assayed. **A.** CFU-GM frequency; **B.** BFU-E frequency; **C.** CFU-MK frequency. ▨ Day 0, □ MGDF+SCF; ■ MGDF+SCF+G-CSF.

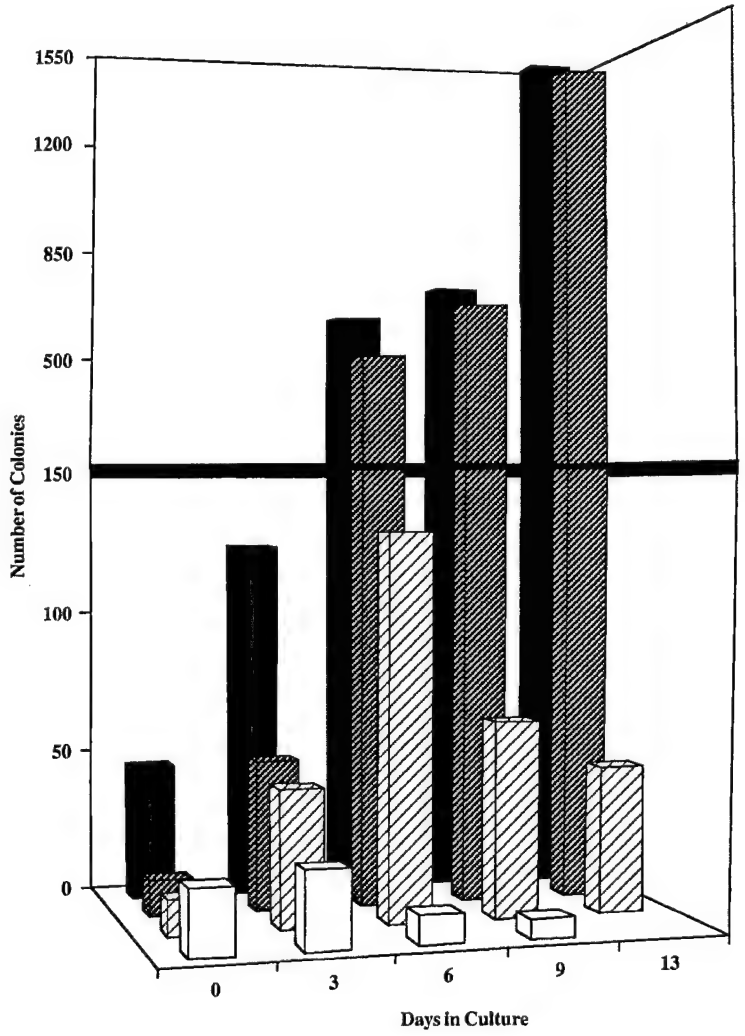

FIG. 5. Absolute CFU-MK numbers. The total number of colonies was calculated from CFU-MK frequency and total cell proliferation. Scale was changed at 150 to reflect the exponential growth of small colonies at day 6 and later. ■ Total colonies; ■ 3–20 cells/colony; ▨ 21–50 cells/colony; □ >50 cells/colony. Notice change of scale of Numbers of Colonies at 150.

in other clinical studies (19–21), we found that the higher seeding density used in this study (4×10^5 cells/ml) improved CFU-MK expansion (by 30% at day 3) and differentiated MK expansion (by 50% at day 13) compared with seeding at only 10^5 cells/ml (data not shown). By adjusting the cell concentration as expansion proceeds, we are able to obtain a greater cell number and MK yield without exhausting the culture medium and possibly harming the cells. Our results using PBMCs from cancer patients are comparable to results published using normal PBMC donors (25), indicating that the patients' underlying cancer has no effect on cell production. In addition, previous studies have shown that the process of $CD34^+$ cell purification does not promote the expansion of any residual tumor cells that may be present in the PBMC harvest (26–29).

The optimal ratio of early MK progenitors versus differentiated megakaryocytes for rapid and sustained platelet engraftment is still not known. Previous studies have found a correlation between the number of infused MK progenitors and rapidity of platelet engraftment (12–14). A threshold number of approximately $1–3 \times 10^5$ MK progenitors/kg patient bodyweight (BW) seems to be required in an autograft for rapid platelet engraftment. Because not every patient can produce this number of MK progenitors, infusing additional *ex vivo*-expanded $CD34^+/CD41^+$ cells may improve platelet recovery.

While it is clear that greater numbers of $CD34^+/CD41^+$ cells infused can induce faster platelet engraftment, it can take over a week for even the most rapidly engrafting PB autograft patients to achieve platelet recovery. *In vivo*, MKs normally have a 16N modal ploidy, and must reach at least 8N ploidy to produce platelets (30,31). *In vitro*, over a week of culture is required for $CD34^+$ cell-derived human MK to achieve ploidy greater than 4N and to begin to produce platelets (32,33). Because patients infused with even the highest numbers of MK progenitors cannot engraft any more rapidly than 1 week, this may indicate that early MK progenitors alone may not be sufficient to induce more rapid engraftment. It may be that a longer period (e.g., 9–13 days) of *in vitro* culture is required to allow megakaryocytes to mature enough to be able to produce platelets soon after infusion. However, infusion of large polyploid cells may increase the risk of blood vessel occlusion, leading to respiratory insufficiency.

Previous attempts to supplement conventional autografts with an *ex vivo*-expanded progenitor cell product produced mixed results as far as platelet engraftment is concerned (19–21). In the first study, Bertolini et al. (19) found that two of the four patients receiving $\geq 2.5 \times 10^5$ $CD61^+$ cells/kg BW, *ex vivo*-expanded for 7 days, did not require platelet transfusions. In this study, MGDF, SCF, IL-3, IL-6, IL-11, Flt-3-ligand, and macrophage inflammatory protein-1α were used to obtain optimal CFU-MK expansion. This is not practical in a clinical setting due to the difficulties associated with obtaining cytokines from multiple corporate sources. In two other studies using a combination of MGDF, stem cell factor, SCF and G-CSF to expand cells for 10 days, Reiffers et al. (20) showed accelerated platelet engraftment, with 11 of 14 patients requiring only one or no platelet transfusions, while McNiece et al. did not (21). Using only MGDF and G-CSF, we have obtained *in vitro* MK productivity equal to or better than that found by Bertolini et al. (19). Wealso confirmed that G-CSF did not improve MK production, but rather favored the myeloid lineage. Moreover, cultures without G-CSF had higher $CD41^+$ frequency than those reported by the Reiffers group (34).

The positive results reported by Bertolini and Reiffers (19,20) clearly indicate that *ex vivo*-expanded MKs can help transplant patients avoid thrombocytopenic platelet nadirs. Possible explanations for this are that the infused cells were: (1) mature MKs, already capable of platelet production, or (2) low ploidy MK progenitors, which rapidly matured *in vivo*. Our results showed that even at day 13 only 7.6% of MKs had a DNA complement greater than 4N. This is in agreement with other reports showing poor *in vitro* MK nuclear maturation (16–32). Thus, it is unlikely that large ploidy MKs are responsible for the rapid platelet engraftment seen by the Bertolini and Reiffers groups (19,20). A more likely explanation is that the megakaryocytes were able to achieve rapid nuclear maturation once infused.

Our goal is to clarify whether early MK progenitors or more differentiated MKs are more beneficial for rapid platelet recovery. We are designing a clinical trial to assess the effectiveness of MKs harvested after different periods of culture and containing different proportions of progenitors and differentiated cells for accelerating platelet engraftment. While greater total cell expansion, containing large numbers of CFU-MK and MKs, were obtained at days 9 and 13, these cells are more mature and differentiated. On the other hand, at days 3 and 6 we found that there was significant expansion of $CD34^+$ and $CD34^+/41^+$ as well as immature CFU-MK, with an increased number of primitive progenitors. We hope to show which product is most conducive to accelerating platelet recovery.

ACKNOWLEDGMENTS

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Appendix III

ELSEVIER

EXPERIMENTAL HEMATOLOGY

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Murine prolactin-like protein E synergizes with human thrombopoietin to stimulate expansion of human megakaryocytes and their precursors

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Objective. **The aim of this study was to determine the effect of promegakaryocytopoietic murine hormone prolactin-like protein E (PLP-E) on human megakaryocytopoiesis.**

Materials and Methods. **Human bone marrow CD34$^+$ cells, cultured in serum-free medium with combinations of thrombopoietin (TPO), stem cell factor (SCF), Flt-3 ligand (Flt-3L), and PLP-E, were analyzed via microscopy, flow cytometry, and clonogenic assay.**

Results. **Unlike the situation with mouse cells, PLP-E alone did not promote human megakaryocyte (MK) differentiation, but instead synergizes with TPO to increase colony-forming unit megakaryocyte (CFU-MK), burst-forming unit erythroid (BFU-E), and and colony-forming unit granulocyte erythroid macrophage mixed (CFU-GEMM) expansion, as well as total MK production. These effects can be attributed to an increase in colony frequency, combined with a significantly greater total cell expansion induced by adding PLP-E along with TPO. The number of cells in each CFU-MK colony is an indication of the maturity of the progenitor population, with larger colonies deriving from a more immature progenitor cell. PLP-E significantly expanded immature, intermediate, and mature CFU-MK subsets at 3 days of culture, as well as the intermediate and mature subsets at day 6. PLP-E combined with TPO induced significant expansion of all CFU-MK subsets at all time points. PLP-E further increased the effect of SCF and Flt-3L on TPO-induced total cell and CFU-MK expansion.**

Conclusions. **PLP-E may act as a survival factor for primitive human megakaryocytic and erythroid progenitors. It appears to preserve the highly proliferative immature fraction of the progenitor compartment but by itself does not promote total cell proliferation or human MK production. PLP-E may prove useful in combination with TPO and other cytokines for ex vivo expansion of hematopoietic progenitors to be used in a clinical setting.** © **2001 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

Keywords: **Prolactin-like protein—Megakaryocytopoiesis—hematopoietic progenitors**

Introduction

Mammalian pregnancy—including that in mice and humans—is characterized by large increases in blood volume and in hematopoiesis. In rodents, megakaryocytopoiesis and platelet levels are elevated throughout gestation [1]. In human pregnancy, platelet production apparently increases to compensate for the dramatic increase in blood volume [2].

Thrombopoietin (TPO) is the primary regulator of megakaryocytopoiesis [3], but it is neither necessary nor sufficient for thrombopoiesis [4–6]. Increased platelet production during pregnancy is likely to result from the presence of higher concentrations of promegakaryocytopoietic cytokines. The placenta is the major endocrine organ of pregnancy in terms of the amount and variety of hormones produced, providing cytokines responsible for the pregnancy-specific alterations in hematopoiesis. In mice, one such cytokine is the placental hormone prolactin-like protein E (PLP-E), a potent cytokine that stimulates murine megakaryocyte (MK) differentiation and the growth of other myeloid cell lineages [7–10]. PLP-E targets mouse MKs through a specific cell surface receptor and induces MK differentiation through a gp130-dependent signal transduction pathway [9].

In vitro, murine megakaryocytopoiesis from cultured hematopoietic cells progresses normally with just TPO treatment and achieves a 16N modal ploidy, similar to in vivo megakaryocytopoiesis [11–14]. Methodology to induce human MK to achieve a normal modal ploidy in vitro has not been described [4,6,15]. Such a goal is important, because

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an agent that specifically enhances human MK production and maturation would be of great clinical use.

To offset posttransplant thrombocytopenia resulting from myeloablative therapy and stem cell rescue and to reduce or eliminate the need for platelet transfusions, methods have been developed for supplementing conventional stem cell transplants with an expanded MK-rich product [16–20]. Because TPO alone lacks the proliferative capacity to expand progenitors or MK in great numbers [21], this factor has been supplemented with pleiotropic early-acting cytokines [6,22]. However, these agents decrease MK frequency and maturation compared to using TPO alone [22,23]. Because cytokines often are functional across species (e.g., TPO), we investigated whether PLP-E might improve human MK yield and thus prove useful in developing methods to expand human MK ex vivo for transplantation.

Materials and methods

Preparation of low-density nonadherent mononuclear cells from peripheral blood aphereses

All samples were collected within the guidelines of the Northwestern University Institutional Review Board on Human Subjects. Bone marrow, obtained from the femur of hematologically normal patients having total hip arthroplasty, was collected in anticoagulant designed to prevent platelet activation and containing final concentrations of 50 IU/mL preservative-free heparin (Life Technologies, Gaithersburg, MD, USA), 1 mM Na_2EDTA, and 0.1 mg/mL DNase I (Boehringer Mannheim, Indianapolis, IN, USA) in 20 mL of Iscove's modified Dulbecco's medium (IMDM; Life Technologies). Marrow cells were repeatedly extracted from bone fragments with IMDM containing 0.1 mg/mL DNase I (Boehringer Mannheim) and 4 μg/mL gentamicin (Life Technologies). The extract was homogenized by passage through a 21-gauge needle to remove bone fragments. Residual red cells were lysed with Ortho-Mune lysing reagent (Ortho Diagnostics, Raritan, NJ, USA) and the remaining cells recovered by centrifugation for 6 minutes at 420*g* through a 10% human serum albumin cushion.

Purification of $CD34^+$ cells

$CD34^+$ cells were purified using magnetic cell sorting (MiniMACS System, Miltenyi Biotec, Auburn, CA, USA) following the manufacturer's recommendations [24]. Cells were passed over two magnetic bead columns and eluted with X-Vivo 20 (BioWhittaker, Walkersville, MD, USA). There were 93.4% ± 1.0% cells determined to be viable using trypan blue (TB) exclusion test, and the average purity was 90.9% ± 1.7% as assessed by flow cytometry.

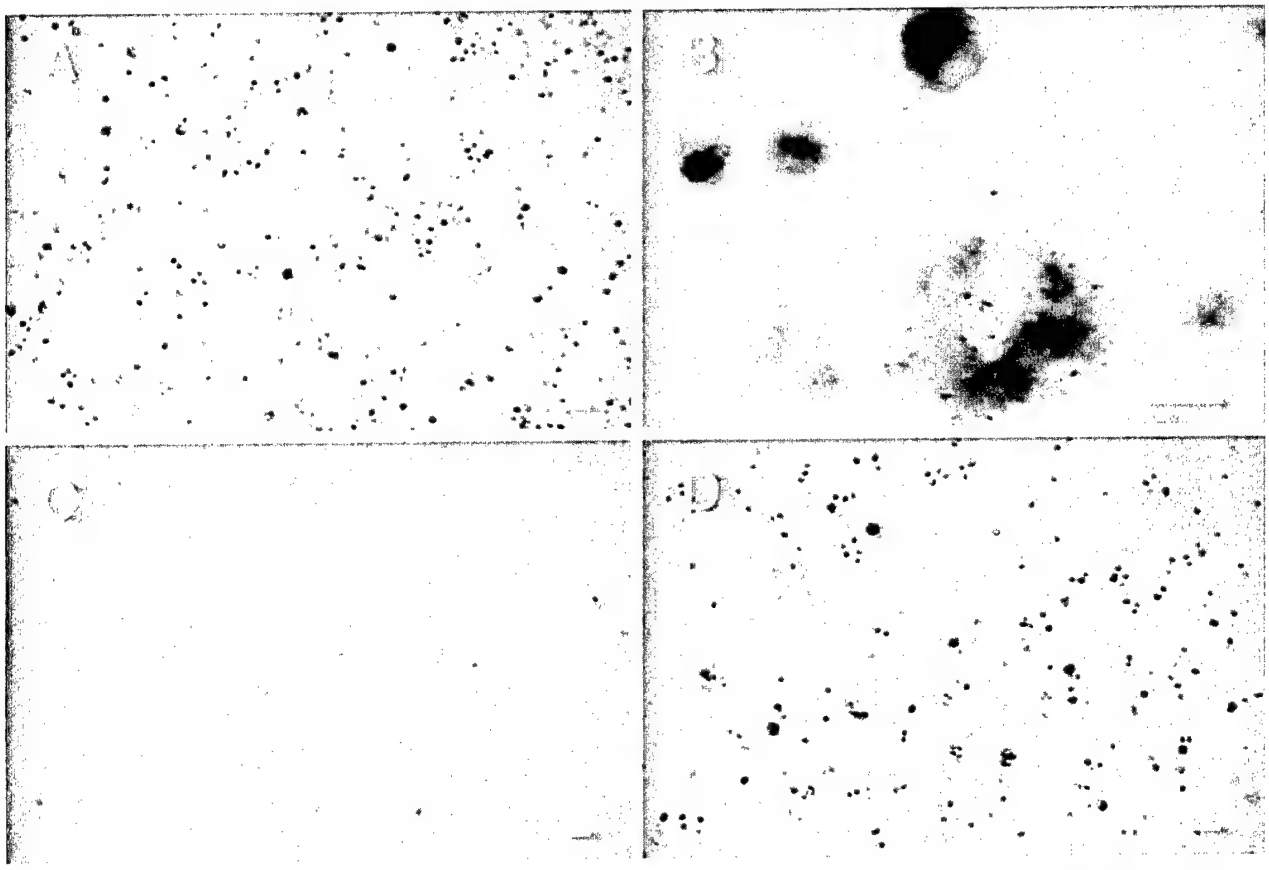

Figure 1. Binding of mouse PLP-E to human bone marrow cells. Purified $CD34^+$ progenitors were cultured in the presence of SCF, TPO, and Flt-3L. Cells were spread onto glass slides and incubated with AP–PLP-E in the absence of competitor **(A,B)** or in the presence of GST–PLP-E **(C)** or GST **(D)**, and stained for AP activity. Note the binding of AP–PLP-E to large differentiated MKs (>20% of cells) and smaller cells **(B)**. Results are representative of three independent experiments. Bar = 100 mm.

Preparation of fusion proteins

The PLP-E cDNA was linked in-frame to a secreted alkaline phosphatase (AP) gene in a mammalian expression vector described previously [9,25]. Fusion protein was obtained by transient transfection of the DNA construct into Chinese hamster ovary cells and subsequent collection of culture medium over a 2-day period. Medium containing secreted AP–PLP-E was concentrated and used in binding assays [9]. PLP-E without the AP fusion partner was generated by similar approaches and used as a competitor in binding experiments. The PLP-E cDNA also was fused to a glutathione S-transferase (GST) coding sequence to produce and purify GST–PLP-E fusion protein from bacteria; GST–PLP-E is biologically active [9].

Culture conditions

For each cytokine combination, 4×10^5 trypan blue-negative (TB^-) cells were seeded per milliliter of serum-free medium. Eleven different bone marrow samples were cultured. GST was used as a negative control in six cultures and was added to TPO in five cultures. Three cultures also had a set of samples treated with TPO, stem cell factor (SCF), and Flt-3 ligand (Flt-3L) with and without PLP-E. Cultures were maintained for 13 days at 37°C in a 5% CO_2 fully humidified atmosphere. TPO, SCF, and Flt-3L were obtained from R&D Systems (Minneapolis, MN, USA), and each was used at a concentration of 100 ng/mL. GST–PLP-E was used at a concentration of 4 μg/mL. Cultures were counted at days 0, 3, 6, 9, and 13 in the presence of TB. If the number of cells per milliliter exceeded 8×10^5, the culture was readjusted to 4×10^5 cells/mL by diluting with fresh medium and cytokines. Only TB^- cells were counted and used for calculating cell proliferation and MK production.

Hormone binding assay

Human hematopoietic cultures (described earlier) were obtained after 9 days in culture. Cells were placed onto glass slides and incubated with or without competitor for 30 minutes at room temperature before incubating with AP–PLP-E for 45 minutes. Binding conditions were as described previously [9]. Slides were washed briefly in Hank's balanced salt solution three times and fixed in a solution containing 20 mM HEPES (pH 7.4), 60% acetone, and 3% formaldehyde. After inactivating endogenous AP at 65°C for 30 minutes, the enzymatic activity derived from the fusion protein was detected by a chromogenic reaction.

Clonogenic assay

Clonogenic assays were performed on six cultures (three with GST controls) on days 0, 3, 6, and 9 according to the manufacturer's instructions. Analysis at day 13 was not done, as preliminary results showed that colony frequency was generally too low to be assayed accurately. Serum-free collagen medium containing TPO, interleukin-3 (IL-3), and interleukin-6 (IL-6; MegaCult-C, Stem Cell Technologies, Vancouver, BC, Canada) was used for colony-forming unit megakaryocyte (CFU-MK) and a serum-free methylcellulose culture medium containing erythropoietin, IL-3, IL-6, SCF, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor (MethoCult GF+, Stem Cell Technologies) was used for colony-forming unit granulocyte-macrophage (CFU-GM), colony-forming unit granulocyte erythroid macrophage mixed (CFU-GEMM), and burst-forming unit erythroid (BFU-E). One thousand cells from each sample at each time point were mixed with the assay medium and plated. CFU-MK colonies were scored after 10 days, and CFU-GM, CFU-GEMM, and BFU-E after 14 days. CFU-MK maturity was evaluated by counting the number of cells in each colony. Three size categories were distinguished: small, mature colonies were those with ≤20 cells; medium-size colonies contained 21–50 cells; and large, immature colonies contained >50 cells.

Flow cytometric analysis

Cell aliquots were washed in phosphate-buffered saline (PBS; Life Technologies) with 5 mM EDTA and 1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO, USA) designed to prevent further platelet activation and/or reverse adherence of activated platelets [26]. After washing, the cells were stained for 15 minutes at 4°C in the dark with phycoerythrin-cyanin 5.1 (PC5)-conjugated-α–CD34 (Clone 581, Coulter-Immunotech, Miami, FL, USA), phycoerythrin (PE)-conjugated-α–CD41 (Coulter-Immunotech), and fluorescein isothiocyanate (FITC)-α–CD15 (Coulter-Immunotech) and analyzed by flow cytometry. Negative controls were PC5-, PE-, and FITC-α–mouse IgG_1 used at equivalent IgG_1 concentrations. Only the nonapoptotic high forward scatter, low side scatter cell population was used for subset analysis.

DNA degradation products (<2N DNA), reflecting apoptosis, and MK ploidy content were measured by flow cytometry. FITC-α–CD41-stained cells were washed again in 1% BSA-PBS and resuspended in 1 mg/mL sodium citrate with 50 μg/mL 7-aminoactinomycin D (Sigma), then incubated for >30 minutes at 4–8°C in the dark [27]. Samples were analyzed using three-color laser. All samples were analyzed on the same day.

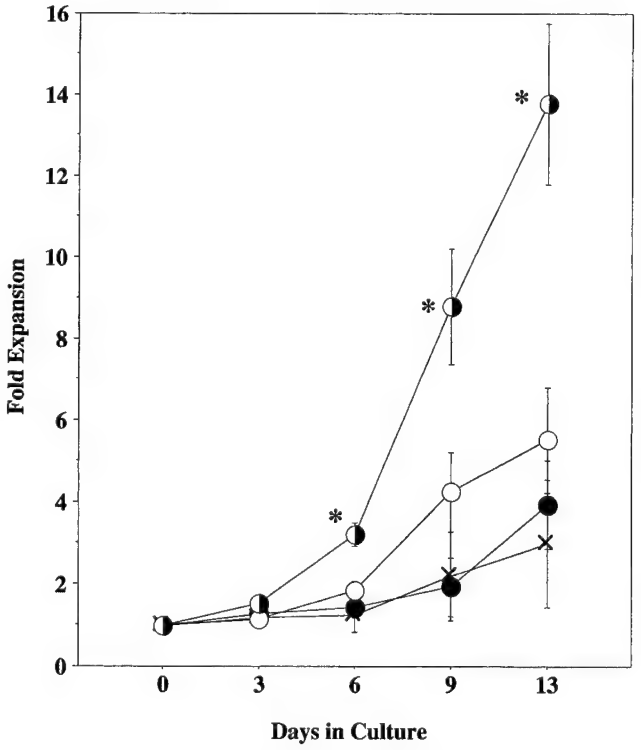

Figure 2. Total viable cell expansion. Purified $CD34^+$ cells were cultured in serum-free medium in the presence of PLP-E (●), TPO (○), PLP-E+ TPO (◑), or GST control (✖). Total TB^- cells were counted at each day and divided by the number of TB^- cells seeded at day 0 to determine the rate of cell expansion. *Significantly greater than TPO. N = 11; GST n = 6.

Statistical analysis

Total cell expansion was normalized per seeded viable (TB^-) cell. All measured cell phenotypes were normalized per seeded $CD34^+$ cell in each respective culture. A Wilcoxon signed rank nonparametric test was applied to each cytokine interaction or each parameter measured. A significant difference was determined for comparisons with $p < 0.05$.

Results

Binding of PLP-E to human hematopoietic cells

PLP-E was found to bind to cells derived from purified $CD34^+$ cells cultured in the presence of TPO, SCF, and Flt-3L (Fig. 1A). PLP-E bound to large, differentiated cells morphologically similar to MK and to unidentified smaller cells (Fig. 1B). The binding of AP–PLP-E was completely eliminated by addition of excess GST–PLP-E (Fig. 1C) but not control GST protein as competitor (Fig. 1D), indicating that the hormone is interacting with a specific and saturable cell surface receptor.

Effect of PLP-E on cell expansion

An effect of PLP-E on human hematopoiesis was assessed first in terms of the growth of primary bone marrow cells in culture. By day 6 in culture and continuing through day 13, the combination of PLP-E and TPO resulted in a synergistic increase in total cell expansion compared to either PLP-E or TPO alone (Fig. 2). Neither TPO nor PLP-E alone increased total cell expansion significantly more than the GST control samples. No difference was seen between TPO samples with or without GST (data not shown). PLP-E+TPO induced sig-

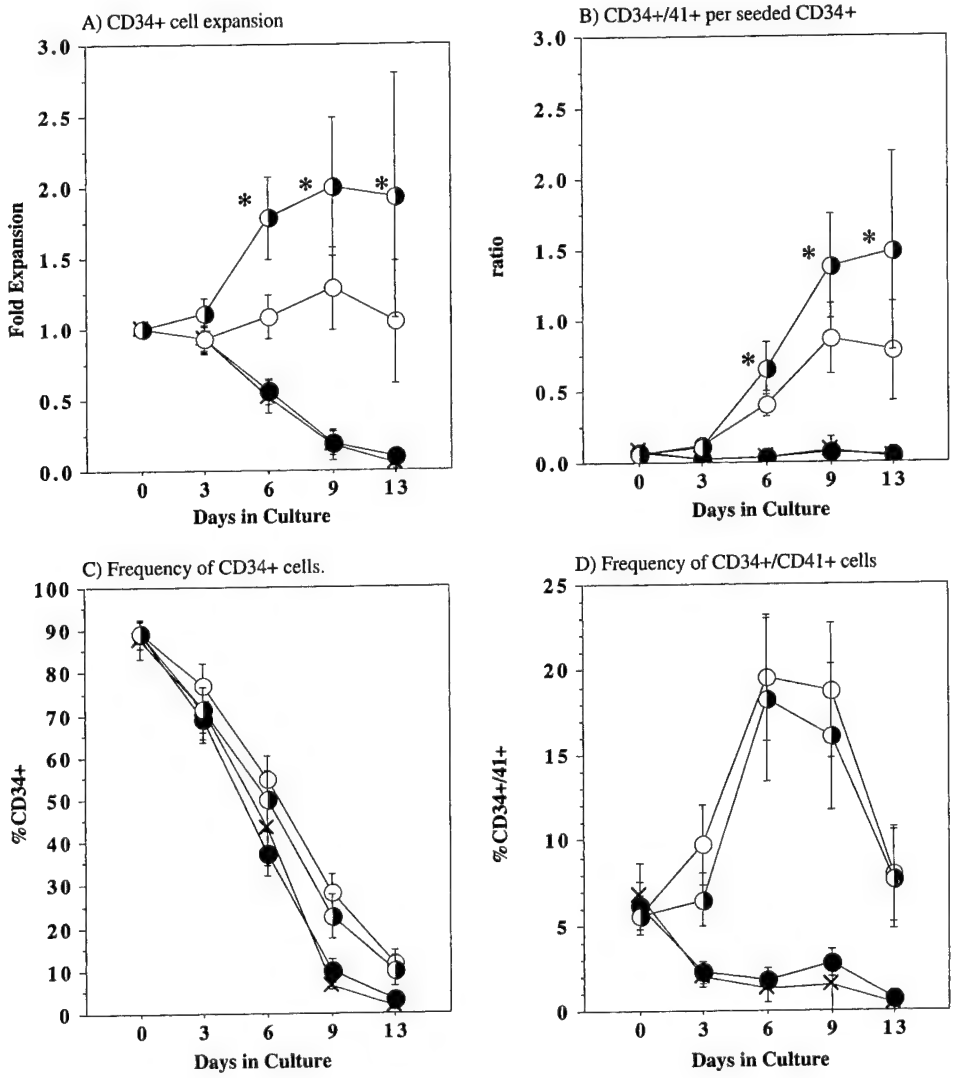

Figure 3. Progenitor cell production. $CD34^+$ cell expansion **(A)** and production of $CD34^+CD41^+$ cells per seeded $CD34^+$ cell **(B)** were obtained by multiplying the total TB^- cell expansion at each day by the frequency of $CD34^+$ **(C)** or $CD34^+CD41^+$ **(D)** cells, respectively, and dividing by the number of $CD34^+$ cells seeded at day 0. PLP-E (●), TPO (○), PLP-E+TPO (◑), or GST control (✖). *Significantly greater than TPO.

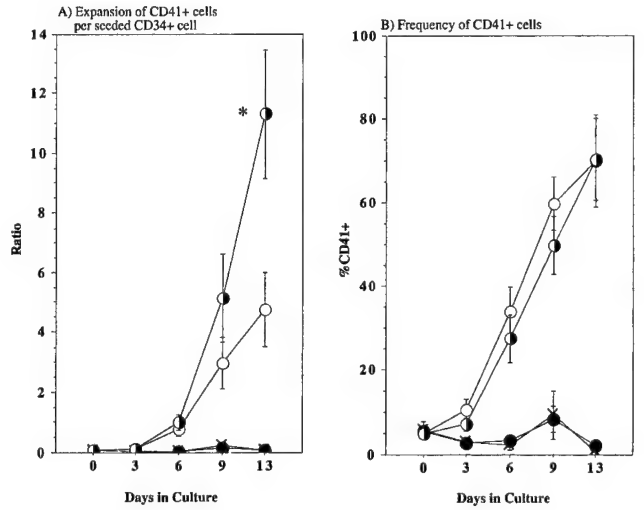

Figure 4. Megakaryocyte production. Production of $CD41^+$ cells per seeded $CD34^+$ cell **(A)** was obtained by multiplying the frequency of $CD41^+$ cells **(B)** cells in the TB^- cell fraction by the total cell expansion and dividing by the number of $CD34^+$ cells seeded at day 0. PLP-E (●), TPO (○), PLP-E+ TPO (◑), or GST control (✖).*Significantly greater than TPO.

nificantly greater $CD34^+$ (Fig. 3A) and $CD34^+CD41^+$ (Fig. 3B) cell expansion by day 6 compared to TPO alone. This increase in progenitor expansion was primarily due to increased cell proliferation (Fig. 2), as the addition of PLP-E to TPO did not change the frequency of $CD34^+$ or $CD34^+CD41^+$ cells in the total cell population compared to TPO alone (Fig. 3C–D).

Effect of PLP-E on MK production

The ability of PLP-E to induce growth of the MK cell population was investigated using CD41 (GPIIb) as a cell surface marker to identify MK cells. PLP-E combined with TPO produced significantly more $CD41^+$ cells per seeded $CD34^+$ cell by day 13 in culture than did TPO alone (Fig. 4A). PLP-E treatment did not alter the percentage of MK cells in the population (Fig. 4B), so again the effect of PLP-E appears to be due primarily to increased cell proliferation.

Colony formation

To determine if PLP-E also acts on progenitor cells to regulate colony-forming activity, cells were transferred to semisolid medium after various days in liquid culture. Compared to controls or TPO alone, the combination of PLP-E and TPO significantly increased the expansion of CFU-MK, BFU-E, and CFU-GEMM at each day measured, but had no effect on CFU-GM expansion beyond the effect of TPO alone (Fig. 5A–D). PLP-E by itself significantly increased the number of CFU-GEMM obtained at 3 days in culture. TPO alone significantly increased CFU-MK frequency and expanded CFU-MK compared to day 0 and compared to control cultures treated with GST. In contrast to the liquid culture results, colony-forming assays also revealed that combining PLP-E and TPO further significantly increased the frequency of CFU-

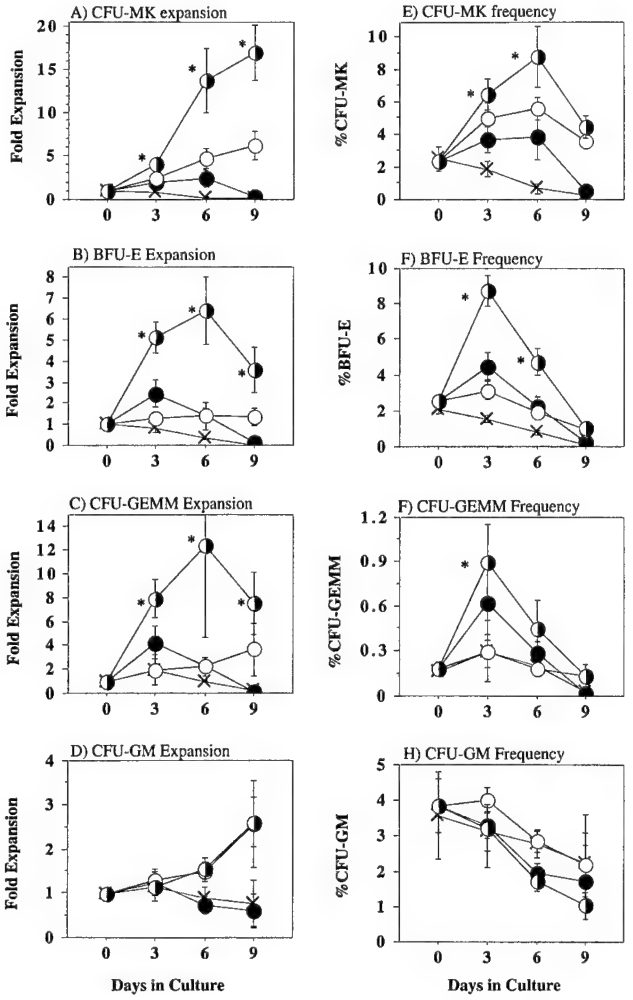

Figure 5. Progenitor colony production. Cells were obtained from samples containing PLP-E (●), TPO (○), PLP-E+TPO (◑), or GST control (✖) at the time of seeding (day 0) or after 3, 6, or 9 days of culture and seeded into collagen-containing serum-free medium (CFU-MK) or methylcellulose (BFU-E, CFU-GM/GEMM). CFU-MK were scored 10 days after seeding, and CFU-GM/GEMM and BFU-E were scored 14 days after seeding. Analysis at day 13 was not done, as preliminary results showed that colony frequency was generally too low to be assayed accurately. Mean expansion was determined by multiplying colony frequency by total cell expansion at the day assayed for that sample. Frequency is the mean number of colonies per 100 total TB^- cells in culture. *Significantly greater than TPO. n = 6; GST n = 3.

MK, BFU-E, and CFU-GEMM, but decreased the frequency of CFU-GM compared to TPO alone (Fig. 5E–H).

CFU-MK maturation

Cells in the MK lineage range from primitive, highly proliferative cells to more mature cells that will soon leave the cell cycle and undergo terminal differentiation. To determine the stage at which PLP-E acts on the MK lineage, CFU-MK colony size was scored. Colonies arising from primitive cells will be larger, because these cells will proliferate extensively before differentiating; in contrast, colonies arising from mature

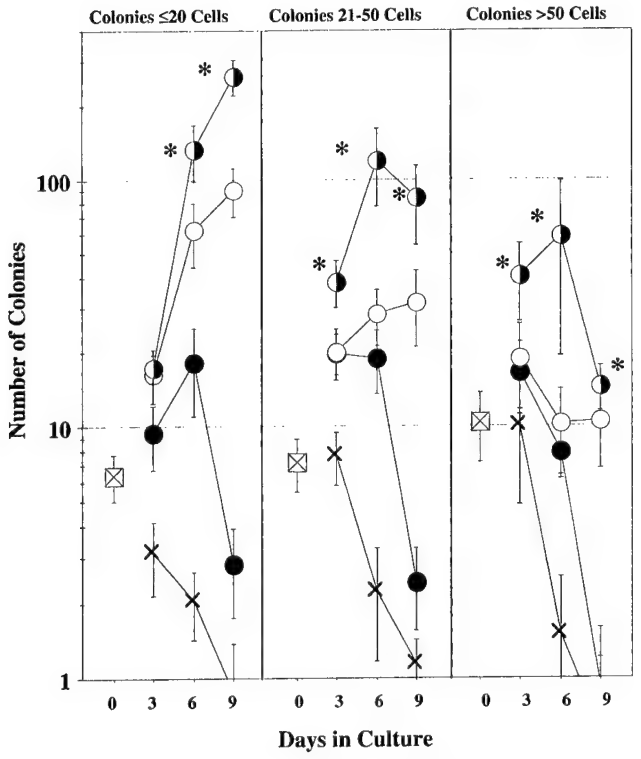

Figure 6. CFU-MK subfraction expansion. CFU-MK obtained in Figure 5 were scored based on colony size. Mean number of mature colonies (CFU-MK producing ≤20 cells), intermediate colonies (CFU-MK producing 21-50 cells), and immature colonies (CFU-MK producing >50 cells) are plotted per 1,000 total cells from cultures containing PLP-E (●), TPO (○), PLP-E+TPO (◑), or GST control (✖) at each sample day. ☒= subfractions at day 0. *Significantly greater than TPO. n = 6; GST n = 3.

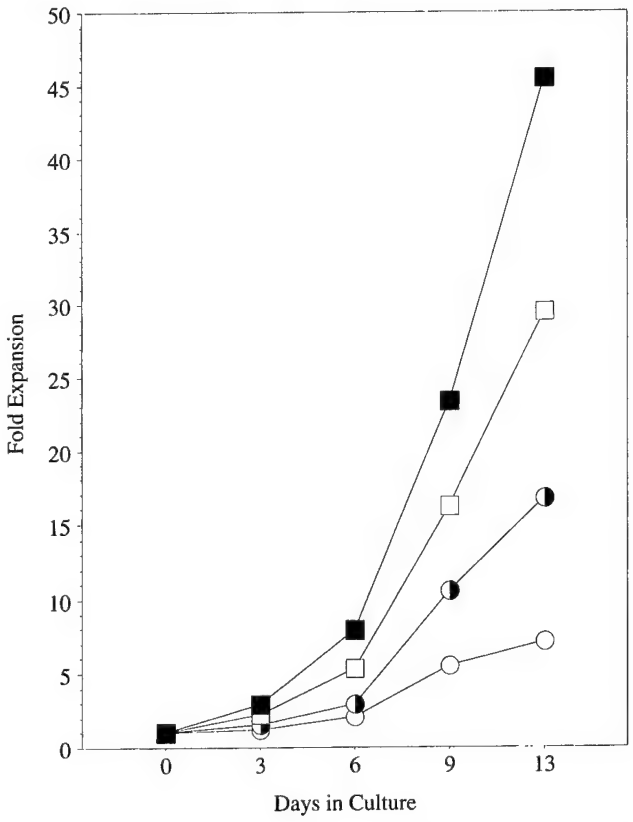

Figure 7. PLP-E increased cell proliferation induced by a progenitor cell cocktail. Purified $CD34^+$ cells were cultured in serum-free medium in the presence of PLP-E+TPO (◑), TPO+SCF+Flt-3L (□), PLP-E+TPO+SCF+Flt-3L (■), or TPO control (○). Total TB^- cells were counted at each day and divided by the number of TB^- cells seeded at day 0 to determine the rate of cell expansion. n = 3.

cells will be small. The initial population of purified $CD34^+$ cells contained 2.4% ± 0.4% CFU-MK. When colonies were assessed for relative maturity by size, 40.4% ± 5.6% of the colonies were derived from primitive CFU-MK based on a colony size >50 cells. Intermediate (21-50 cells/colony) and mature CFU-MK (<20 cells) represented 28.9% ± 2.6% and 30.7% ± 7.7% of the seeded CFU-MK, respectively.

In cultures treated with PLP-E alone, all three CFU-MK subsets expanded at day 3, as well as the mature and intermediate subsets at day 6 compared to day 0 (Fig. 6). TPO-only treatment gave similar results, and TPO maintained an expanded number of mature and intermediate CFU-MK subsets at day 9. When cells were treated with PLP-E+TPO, all three subsets expanded significantly more than with TPO or PLP-E alone, and a significant total expansion of the primitive subset of CFU-MK was seen at days 6 and 9 compared to day 0. GST-treated control samples failed to produce any CFU-MK.

PLP-E performance in a progenitor cell expansion cocktail

TPO, SCF, and Flt-3L (long-term culture-initiating cell [LTC-IC] cocktail) are an optimal cytokine combination for the promotion of LTC-IC [28,29]. LTC-IC cocktail induced greater total cell expansion than PLP-E+TPO, and adding PLP-E to the LTC-IC cocktail had an additive effect on total cell proliferation (Fig. 7). SCF and Flt-3L also decreased $CD34^+CD41^+$ and $CD41^+$ frequency (Fig. 8), but PLP-E had no effect, similar to data shown in Figures 3D and 4B. However, PLP-E increased CFU-MK frequency when added to TPO, whereas SCF and Flt-3L decreased CFU-MK frequency (Fig. 9A). Thus, although SCF and Flt-3L did not increase the overall expansion of CFU-MK compared to TPO alone, PLP-E significantly increased it overall and synergized with SCF and Flt-3L to increase CFU-MK nearly four-fold compared to TPO at day 9 (Fig. 9B).

Discussion

We now have found that a recently described murine cytokine, PLP-E, contributes to the ex vivo expansion of the human MK and erythroid lineages through effects on CFU-MK, BFU-E, and their common precursor CFU-GEMM. The effect of PLP-E augments the action of other cytokines, such as TPO, SCF, and Flt-3L. PLP-E alone transiently in-

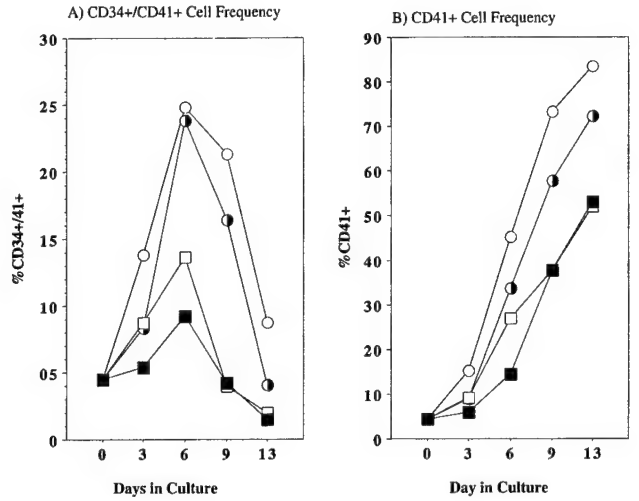

Figure 8. Decreased $CD34^+CD41^+$ and $CD41^+$ frequency induced by a progenitor cell cocktail. Purified $CD34^+$ cells were cultured in serum-free medium in the presence of PLP-E+TPO (◑), TPO+SCF+Flt-3L (□), PLP-E+TPO+SCF+Flt-3L (■), or TPO control (○). Frequency of **(A)** $CD34^+CD41^+$ cells or **(B)** $CD41^+$ cells in TB^- cells at each day as assayed by flow cytometry. n = 3.

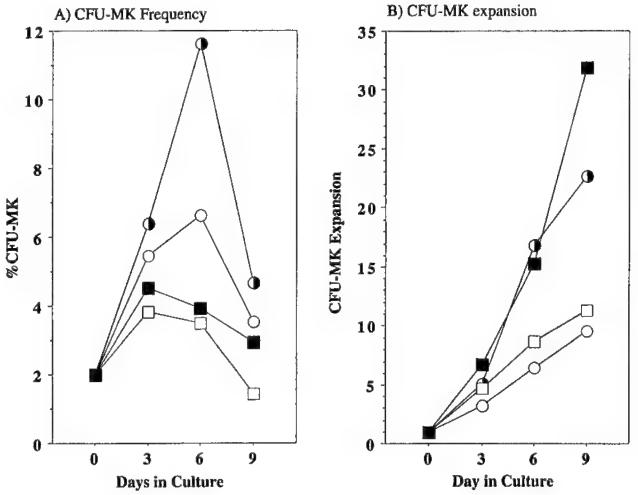

Figure 9. CFU-MK production induced by LTC-IC cocktail. Cells were obtained from PLP-E+TPO (◑), TPO+SCF+Flt-3L (□), PLP-E+TPO+SCF+Flt-3L (■), or TPO control (○) samples at the time of seeding (day 0) or after 3, 6, or 9 days of culture, seeded into serum-free collagen, and then scored 10 days after seeding. **(A)** Frequency is the mean number of colonies per 100 total cultured cells. **(B)** Mean expansion was determined by multiplying colony frequency by total cell expansion at the day assayed for that sample. n = 3.

creased the frequency and number of CFU-GEMM and immature CFU-MK in populations derived from purified $CD34^+$ precursors. PLP-E in combination with TPO results in a synergistic expansion of total cells, primitive progenitors, and MK cells.

PLP-E induces murine MK differentiation through a gp130-dependent signal transduction pathway [9], and it is known that cytokines that signal via the gp130 receptor subunit may synergize with TPO to enhance megakaryocytopoiesis in both mice [14] and humans [30]. PLP-E, like TPO, appears to promote the survival of the most primitive MK progenitors, with TPO then acting to stimulate further growth and differentiation, especially of the MK lineage. In this way, PLP-E synergizes with TPO to expand MK. This interpretation is consistent with the greater number of large, immature CFU-MK in the presence of PLP-E and with the observed increase in total cell proliferation without a substantial decrease in $CD34^+CD41^+$ cell frequency when PLP-E and TPO are combined.

When scoring the effect of PLP-E by itself on the total $CD34^+$ population in liquid culture, no response was seen in terms of proliferation, and no increase was detected in megakaryocytopoiesis. The inability of PLP-E by itself to promote human MK cell differentiation, whereas PLP-E stimulates increases in mouse MK cell size and ploidy [9], may result from differences in the systems used for human and mouse hematopoietic cell culture. It also is conceivable that human and mouse MK cell biology differ sufficiently to account for distinct responses to this hormone. Murine MK cells are capable of achieving a normal modal ploidy of 16N in culture [11,12,14], whereas human MK cells are not [4,6].

Although no PLP-E homologue is known in humans, the specific binding of PLP-E to human hematopoietic cells indicates that the unidentified PLP-E receptor may be functionally conserved between mouse and human species. Thus, humans may produce either a PLP-E that remains to be identified or a distinct ligand for the putative PLP-E receptor. It certainly is possible that PLP-E shares its receptor with other ligands, even within the mouse, because PLP-E is only detected in pregnant females, whereas the PLP-E receptor also is expressed in MK cells in nonpregnant female and male mice [9]. If the PLP-E receptor does bind multiple ligands, these ligands appear to be unknown. Binding studies using numerous other cytokines with megakaryocytopoietic activity have revealed no competition with PLP-E for receptor occupancy (data not shown).

PLP-E provides a means of enhancing the activity of currently available cytokine cocktails used to promote hematopoietic progenitor cell growth in culture. When compared to other early-acting cytokines such as SCF and Flt-3L in combination with TPO, PLP-E appears to target more specifically the MK compartment, resulting in a purer MK preparation. This effect may prove to be a significant advantage in ex vivo MK expansion for use in a clinical setting. That this mouse placental hormone also is capable of binding to human hematopoietic cells and stimulating their growth attests to the potential clinical value of identifying and characterizing new cytokine activities in model systems, especially when human homologues are not known (as is the case for PLP-E).

Acknowledgments

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Appendix IV

Fibronectin- and protein kinase C–mediated activation of ERK/MAPK are essential for proplateletlike formation

Fang Jiang, Yuzhi Jia, and Isaac Cohen

The megakaryoblastic CHRF-288 cell line was used to investigate signal transduction pathways responsible for proplateletlike formation (PPF). The role of fibronectin (FN) and protein kinase C (PKC) activation in PPF were examined. In the presence of serum and phorbol 12-myristate 13-acetate (PMA), a PKC activator, cells exhibited full megakaryocytic differentiation, manifested by adhesion, shape change, increased cell size, polyploidy, PPF, and expression of CD41$^+$, CD61$^+$, and CD62P$^+$. The same morphologic and phenotypic features were observed in serum-free cultures in the presence of FN/PMA. Only partial differentiation occurred when other integrin ligands were substituted for FN. FN alone induced minimal cell adhesion and spreading, while PMA alone induced only polyploidy without adhesion. Signal transduction changes involved the activation of the extracellular signal–regulated protein kinase 1 (ERK1)/ERK2 as well as c-Jun amino-terminal kinase 1 (JNK1)/stress-activated protein kinase (SAPK). Phosphoinositide-3 kinase and p38 were not stimulated under these conditions. Inhibitors were used to identify the causal relationship between signaling pathways and PPF. PD98059 and GF109203X, inhibitors of ERK1/ERK2 pathway and PKC, respectively, blocked PPF, while adhesion, spreading, and polyploidy were normal. These studies show that activation of ERK1/ERK2 mitogen-activated protein kinase pathway plays a critical role in PPF. The elucidation of the signal transduction pathway on megakaryocyte development and PPF is of crucial importance for understanding this unique biological process. (Blood. 2002;99:3579-3584)

Introduction

Megakaryocytopoiesis involves proliferation of megakaryocyte (MK) progenitors and differentiation. Following a few mitotic cycles, an aborted mitotic process takes place in which prophase, metaphase, and initial anaphase stages are normal while late anaphase and telophase are deficient.[1,2] Cytokinesis does not take place, and endoreplication cycles lead to polyploidy.[3] Nuclear maturation proceeds in concert with cytoplasmic differentiation and expression of megakaryocytic markers. Cytoplasmic maturation occurs at different ploidy levels and results in platelet production (thrombopoiesis). There are different hypotheses pertaining to platelet formation and release from mature MKs. In one model, the demarcation membrane system outlines nascent platelets derived from the interior of the cytoplasm.[4,5] Tubular-branching demarcation membranes may be reorganized by a process of fusion-fission into flat sheets of membrane with subsequent shedding of platelets into the circulation.[6] In another model, platelets are thought to be released from extruded long cytoplasmic extensions by rupture of the slender links between them.[7-9] Proplateletlike formation (PPF) in vitro follows cell adhesion and polyploidization.[10,11]

Protein kinase C (PKC) is a family of serine/threonine protein kinases in the cytosol involved in pleiotropic processes such as cell growth, differentiation, and cytokine secretion.[12,13] The role of PKC signaling in MK differentiation has been well established over the past 2 decades. In several cell lines, PKC activation by the agonist phorbol 12-myristate 13-acetate (PMA) induced MK differentiation, including cell cycle arrest, secretion of cytokines, up-regulation of MK surface antigens, polyploidization, development of proplatelet processes, and appearance of demarcation membranes.[14-16] Integrins, which are heterodimeric transmembrane protein receptors, mediate cell membrane–extracellular matrix interaction and have profound effects on cell division, differentiation, and survival.[17] It has become clear that integrins not only mediate the physical attachment of cells to extracellular matrix but also generate a variety of signals to the interior of the cell[18-20] and regulate cell growth, survival, and gene expression.[21-24] Integrin-mediated cell adhesion has been shown to strongly activate mitogen-activated protein kinase (MAPK), a key downstream effector of the Raf signaling pathway in Swiss 3T3/REF52 fibroblasts[25] and NIH3T3 fibroblasts.[26-27] PKC activation and integrin engagement signaling pathways play different roles in physiological processes and may generate cross-talk resulting in either up-regulation or down-regulation, according to the particular demands of different cells.

MAPKs are serine/threonine kinases that are highly conserved in eukaryotic cells from yeast to human. Several signaling cascades classified as MAPK pathways include extracellular signal–related kinase kinase (MEK)–extracellular signal–regulated protein kinase 1 (ERK1)/ERK2; MEK–c-Jun amino-terminal kinase (JNK)/stress-activated protein kinase (SAPK); and MEK-p38. These pathways have been identified as mediating cell proliferation, survival, differentiation, and apoptosis. While the ERK pathway responds

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mainly to mitogens and growth factors and regulates mammalian cell proliferation and differentiation, the JNK/SAPK MAPK pathway is associated with stress and apoptosis.[28] The p38 MAPK pathway is activated in response to cellular stress and inflammation and is involved in many fundamental biological processes.[29-31]

Phosphoinositide-3 kinase (PI3K) is known to initiate several signaling pathways,[32] and recently, it has also been found to stimulate the activation of the ERK1/ERK2 MAPK cascade in some signaling systems as well. Cross-talk and signal integration mechanisms among all these pathways have been described.

While primary MKs have been separated by different methods,[33-36] the yield and degree of purity of the cells are relatively poor, and consistent PPF is lacking. The study of human megakaryocytopoiesis and thrombopoiesis requires the development of either long-term culture systems derived from normal MKs or permanent cell lines derived from transformed MKs. Therefore, for the purpose of clarifying the mechanism/signal transduction of PPF, we chose a human megakaryoblastic CHRF-288 cell line as a culture model that has proved to be valuable for the study of MKs and MK-associated functions.[37] Our results showed that PPF in CHRF-288 cells needed 2 critical steps: fibronectin (FN) binding to the cell and activation of intracellular PKC by PMA. The activation of the MEK/ERK pathway induced by FN binding and the activation of PKC are necessary for PPF. Our results suggest substantial overlaps and cross-talk between the integrin engagement pathway, PKC activation, and the Ras/MEK/MAPK cascade.

Materials and methods

Materials

The human CHRF-288 megakaryoblastic cell line was generously provided by Dr Fern Tablin (Davis, CA) and Dr Michael Lieberman (Cincinnati, OH). PMA, extracellular matrix proteins, and BCIP (5 bromo-4-chloro-3-indolylphosphate)/nitroblue tetrazolium (NBT) liquid substrate system were from Sigma (St Louis, MO). Thrombopoietin (TPO) was from R&D (Minneapolis, MN). Proteinase inhibitors were from Boehringer Mannheim (Germany). Tris-glycine gel was from Gradipore (Frenchs Forest, New South Wales, Australia). Nitrocellulose membrane was from Amersham (Les Ulis, France). ERK1/ERK2 MAPK antibody, phosphorylated ERK1/ERK2 MAPK antibody, and goat antirabbit immunoglobulin G (IgG) alkaline phosphatase conjugate were from Promega (Madison, WI). The p38 MAPK antibody and phosphorylated p38 MAPK antibody, the JNK/SAPK antibody and phosphorylated JNK/SAPK antibody, and the PI3K/Akt antibody and phosphorylated PI3K/Akt (Ser and Thr) antibodies were from New England Biolabs (Beverly, MA). Signaling pathway inhibitors PD98059, SB203580, Wortmannin, LY294002, and GF109203X were from Calbiochem (San Diego, CA).

Cell culture

CHRF-288 cells were cultured in Fischers medium (Sigma) supplemented with 20% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD), as a source of growth factors and integrin ligands, and 4 mg/L gentamicin. To identify the serum factor(s) responsible for MK differentiation, serum-free culture was carried out with X-vivo 20 culture medium (Biowhittaker, Walkersville, MD)[38] in plates coated with extracellular matrix proteins, such as FN, vitronectin (VN), laminin (LN), collagen I (CI), and collagen IV (CIV). Poly-D-lysine and bovine serum albumin (BSA) were used as controls. The coating procedure involved dissolving the matrix protein in phosphate-buffered saline (PBS) at a concentration of 50 μg/mL and using it in 12-well culture plates (200 μL per well). Plates were left at 4°C overnight, washed with PBS, and incubated with a blocking agent (1% BSA) for 2 hours at 37°C before the onset of culture. PMA (10 ng/mL) was used to promote cell differentiation. The model culture system for PPF included X-vivo 20 plus FN plus PMA. When inhibitors of signaling pathways were used, they were added to the culture 1 hour prior to PMA addition.

Morphologic assay

Differentiated cells were counted by means of micrometer grids in 1-mm^2 areas. Under the 10 × objective lens, the left edge of each culture well was used as the first field; the adjacent 5 fields were counted one by one. Adherent and differentiated cells were stained in situ with Wright Giemsa stain for 15 to 30 minutes, or photographed directly with an LSM510 laser microscope (Zeiss, Jenna, Germany).

Electron microscopy

Cells or plateletlike particles were fixed in 2% glutaraldehyde, washed with cacodylate buffer, postfixed with 1% osmium tetroxide and uranyl acetate, dehydrated in graded alcohol and propylene oxide, and then embedded with Epon. Ultrathin, 60-nm sections were cut, stained with uranyl acetate and lead citrate to enhance contrast, and examined in a JEOL-100CX electron microscope (Tokyo, Japan).

Flow cytometry for phenotypic analysis

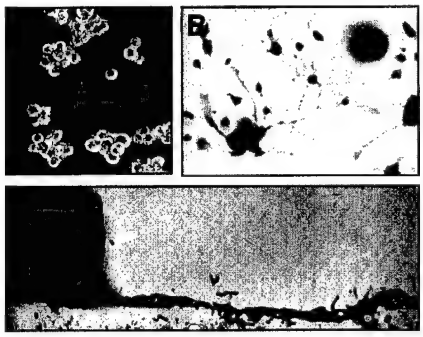
Cells were counted and viability was assessed by the trypan blue exclusion method. For flow cytometry, cells were washed in PBS containing 1% BSA and then stained for 15 minutes on ice in the dark with fluorescent dye–conjugated monoclonal antibodies specific for MK markers (CD41, CD61, and CD62P) or early myeloid cell markers (CD33 and CD90) (Beckman Coulter, Brea, CA). Corresponding negative controls were fluorescein isothiocyanate–antimouse IgG, phycoerythrin-antimouse IgG, and Cy5-antimouse IgG (Beckman Coulter) used at equivalent IgG concentrations. Each sample contained 5×10^5 cells. Flow cytometric analysis was performed by means of a Coulter Cytometry XL dual laser flow cytometer (Coulter, Hialeah, FL).

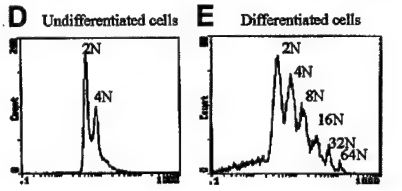
Ploidy analysis

Nonadherent cells were collected by centrifugation. Adherent cells were first dislodged with 0.05% trypsin in 0.33 mM EDTA, and the viable cells counted by trypan blue exclusion. Then, 5×10^5 cells per sample were washed in PBS containing 1% BSA. DNA was stained with 7-amino-actinomycin D (7-AAD) (Calbiochem) following a one-step fixation-permeabilization with the ORTHO PermeaFix reagent (J & J, Raritan, NJ). The ploidy classes were then determined following flow cytometric analysis.

Protein extraction and Western blot analysis

Cells were collected at 5 minutes, 30 minutes, 1 hour, 2 hours, 24 hours, 48 hours, and 72 hours after PMA treatment in different culture conditions. Nonadherent and dislodged adherent cells were centrifuged at 400*g* for 5 minutes, and the cell pellets lysed in a buffer containing 30 mM Hepes, 100 mM NaCl, 10 mM benzamidine, 1 mM EDTA, 1% Triton-X-100, and 20 mM NaF, adjusted to pH 7.5. The following protein and phosphatase inhibitors were then added: 1 mM phenylmethyl sulfonyl fluoride, 10 μg/mL aprotinin, 5 μg/mL leupeptin, 2 μg/mL pepstatin, 1 mM Na_3VO_4. Cell lysates were cleared by centrifugation at 16 000 rpm for 5 minutes at 4°C. The protein concentrations were measured by means of protein/DC Assay (Bio-Rad, Hercules, CA) to ensure equal electrophoretic loading. Lysates were denatured by boiling for 5 minutes in Laemmli sample buffer and loaded at a concentration of 20 μg protein per lane on 10% Tris-glycine iGel. Proteins were then transferred to nitrocellulose membranes and blocked for 16 hours in Tris-buffered saline containing 0.05% Tween 20 and 1% BSA. To ensure equal protein loading, duplicate gels were stained by Ponceau S (Sigma). Antibodies against signal proteins or phosphorylated signal proteins were used to determine the activation of the kinases during cell differentiation. Each antibody was diluted in blocking buffer at the concentration recommended by the supplier and incubated with the blot at room temperature for 2 hours or at 4°C overnight, then incubated with alkaline phosphatase conjugate for 1 hour at room temperature, and color-detected by BCIP/NBT substrate liquid system.





	2N	4N	8N	16N	32N	64N
Undifferentiated Cells %	49.5	32.4				
Differentiated Cells %	26.8	24.2	16.9	9.35	4.18	1.44

Figure 1. Cell morphology in serum-containing culture medium. Cells were cultured for 3 days. (A) Undifferentiated cells cultured in 20% FBS-containing medium. Original magnification × 200. (B) PMA-induced full differentiation in serum-containing medium, showing adherence, size increase, polyploidization, and proplatelet formation. In situ Giemsa staining. Original magnification × 200. (C) Electron micrograph showing a differentiated cell with an elongated cytoplasmic process. Original magnification × 3000. (D) Ploidy of undifferentiated cells. (E) Ploidy of differentiated cells. Nonadherent cells and adherent cells (dislodged by means of 0.05% trypsin-EDTA) were collected by centrifugation. Cells were stained with 7-AAD as indicated in "Materials and methods" and analyzed by flow cytometry. Representative of 5 independent experiments.

Statistical analysis

Statistical analysis was performed by means of the 2-tailed Student *t* test for unpaired data in phenotypic, ploidy, and morphologic analysis.

Results

CHRF-288 cell differentiation in serum-containing medium

In the absence of PMA stimulation in serum-containing culture medium, CHRF-288 cells were nonadhesive and proliferated actively (Figure 1A). However, after 5 minutes of exposure to PMA (10 ng/mL), they gradually underwent full differentiation, including adhesion, spreading, size increase, polyploidization, and PPF (Figure 1B), reaching maximal differentiation after 3 days. Thereafter, cells detached from the substrate and underwent apoptosis and cell death. The cells with PPF were characterized by electron microscopy. These elongated cytoplasmic processes (Figure 1C) contained cytoskeletal structures, mitochondria, rough endoplasmic reticulum, macrovesicles, and α-granule–like organelles, but no microtubules were observed. Similar morphology was observed for the plateletlike particles, and no marginal microtubular rings, typical of platelets, were seen. The particles did not aggregate with adenosine 5′-diphosphate or thrombin. The timing of the differentiation stages was dependent on PMA concentration (1 ng/mL, 10 ng/mL, or 100 ng/mL). The higher the PMA concentration, the more rapid the differentiation pattern. Without PMA addition, cells showed few adhesion and shape change patterns (Figure 1A). TPO had no effect on the differentiation of CHRF cells.

PMA treatment altered the cell surface expression of some membrane proteins (Table 1). $CD33^+$, $CD90^+$, and $CD62P^+$ cells significantly decreased, while $CD41^+$ and $CD61^+$ did not change. Although cultured CHRF cells show a 2N-4N ploidy pattern in the absence of PMA (Figure 1D), the addition of PMA led to a significant increase of the DNA content and ploidy distribution, with the appearance of 4N to 64N polyploid cells (Figure 1E).

FN is the integrin ligand responsible for full differentiation

Different integrin ligands were substituted for serum in the culture medium. While FN alone induced minimal adhesion and shape change, PMA alone induced increased size and polyploidy in the absence of adhesion; FN and PMA costimulation induced full cell differentiation/PPF (Figure 2A). In this culture system, the morphologic features of PPF and functional tests of the particles were similar to the data obtained in serum-containing culture. PMA-treated cells cultured in the presence of LN increased in size without adhesion or PPF. PMA-treated cells cultured in the presence of VN-, CI- and CIV-coated wells showed minimal adhesion and PPF (Figure 2B). No adhesion or differentiation occurred with either BSA or poly-D-lysine–coated culture wells. We determined that the optimal culture model for differentiation and PPF involves seeding CHRF cells at a concentration of 2.5 to 5×10^4 cells per milliliter in X-vivo 20 in the presence of PMA (10 ng/mL) in FN-coated dishes for 3 to 4 days. Using this culture model in a well-defined medium resulted in more consistent PPF than the use of serum in the culture medium.

Activation of ERK1/ERK2 is essential for full differentiation and PPF

The differentiation of CHRF cells can be viewed as the result of a balance between stimulatory and inhibitory signals. Western blots were used to identify the activation of signaling molecules related to PPF. When cells were treated with FN alone, a faint phosphorylation signal of ERK1/ERK2 was detected after 1 day of culture (Figure 3A, upper panels). With PMA alone, on the other hand, a weak and quick phosphorylation signal of ERK1/ERK2 was detected within 5 minutes and diminished with time (Figure 3A, middle panel). With both FN and PMA present in the culture, a strong phosphorylation of ERK1/ERK2 occurred. The activation of ERK1/ERK2 was rapid, occurring within 5 minutes of exposure to PMA. The maximum expression of phosphorylated ERK1/ERK2 occurred 30 minutes after PMA treatment, when the cells had firmly adhered to the FN substrate. PMA rapidly and persistently induced the phosphorylation of ERK1/ERK2, but the expression of the kinase tapered with time (Figure 3A, lower panel). Use of the inhibitors PD and GFX in the culture system blocked phosporylation of ERK1/ERK2, as did a high dose of SB (50 μM) (Figure 3B).

Table 1. Immunocytochemical characteristics

Antigen cluster designation, % reactivity	Undifferentiated cells, %	Differentiated cells, %	*P* value
CD33	99.5	81.3	< .01
CD41	72.5	81.2	.39
CD61	54.4	63.9	.33
CD62P	49.6	25.3	.01
CD90	93.4	61.8	< .01

Data are from a flow cytometric analysis of cultured CHRF-288 cells. Cells stained with specific surface antibody conjugated with fluorescent dye as indicated in "Materials and methods." The percentages are percentages of the total number of cells. Five independent experiments were performed.

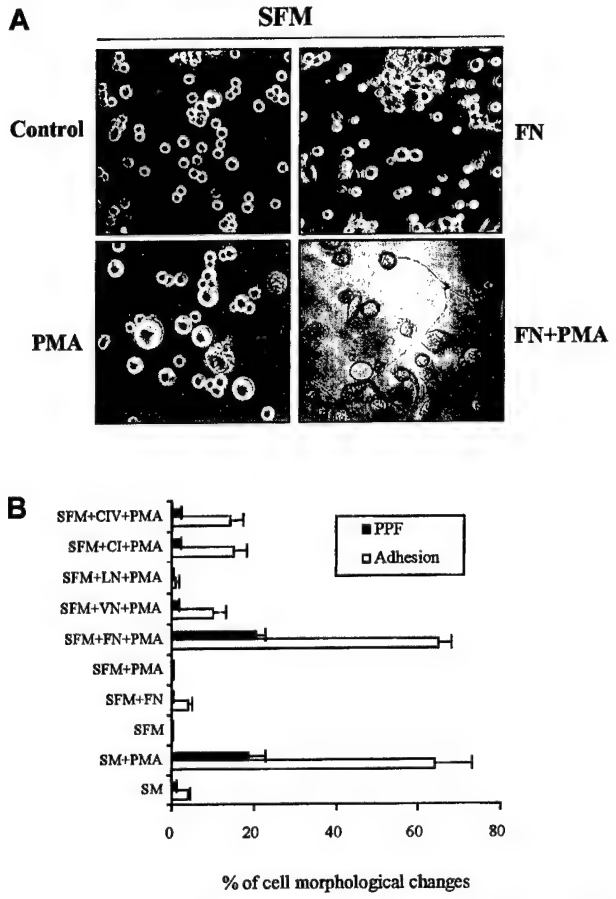


Figure 2. Effect of single matrix protein on CHRF cell morphology with or without PMA treatment in serum-free medium culture. (A) Cells cultured in serum-free medium with different combinations. Full cell differentiation, including PPF, occurred only in FN/PMA costimulation. Original magnification × 200. (B) Culture dishes were coated with a single matrix protein (50 μg/mL) overnight. Cells were cultured in serum-free medium in the presence of PMA (10 ng/mL) and a single matrix protein for 3 days. Cells were seeded at a concentration of 2.5×10^4 cells per milliliter and counted in 1-mm² areas by means of phase contrast microscopy. SM, serum-containing medium; SFM, serum-free medium. Graph depicts the means ± SE from at least 5 experiments.

To determine a causal relationship between signaling and PPF, we supplemented our culture model system with various signaling inhibitors to identify the pathway responsible for differentiation of the CHRF-288 cells (Figure 4). The inhibitors were added to cells 1 hour before PMA addition. PD98059 (10 μM and50 μM) and GF109203X (5 μM), inhibitors of upstream of ERK1/ERK2-MAPK and PKC,[39] respectively, blocked PPF but not adhesion or size increase. While a low dose of SB203580 (10 μM), an inhibitor of p38 MAPK[40] and JNK/SAPK[41] pathways, did not affect cell differentiation, a high dose (50 μM) of SB blocked the differentiation. Wortmannin (100 nM and 500 nM), an inhibitor of PI3K/Akt pathway,[39] did not affect cell differentiation. A high dose of GF109203X (25 μM) was cytotoxic for cells as determined by the trypan blue exclusion test, and the cell body shrank.

Activation of JNK1/SAPK but not p38 and PI3K

Costimulation of cells with FN/PMA induced phosphorylation of JNK1(46 kd)/SAPK within 5 minutes of PMA addition, and this activation lasted at least 2 hours (Figure 5A). Low and high doses of SB (10 and 50 μM) blocked phosphorylation of JNK1 (Figure 5B), but only a high dose of SB blocked PPF (Figure 4C). JNK1/SAPK is therefore not involved in PPF. Neither p38 MAPK nor PI3K/Akt (Ser473 and Thr308) were activated following FN/PMA stimulation (data not shown).

Discussion

The identification of the signaling pathway responsible for megakaryocytic differentiation, PPF, and platelet release is still debated. In this study, we sought to characterize the MAPK-specific targets in CHRF-288 cells stimulated by FN/PMA. Rapid and prolonged activation of ERK1/ERK2 MAPK was found necessary for PPF.

We first investigated a series of integrin ligands for their effects on PPF.[17] As a main component of matrix protein, FN binds to several megakaryocyte integrins, such as $\alpha IIb\beta_3$, $\alpha v\beta_3$, $\alpha v\beta_5$, and $\alpha 5\beta_1$. In this study, when CHRF cells were cultured in serum-free medium in FN-coated dishes, only later-term and weak phosphorylation of ERK1/ERK2 and minimal adhesion were observed. With the addition of PMA, a PKC activator used at a moderate concentration that did not down-regulate PKC,[42] the cells showed a dramatic full differentiation pattern that included PPF, and concomitant activation of ERK1/ERK2. The binding to other matrix proteins, VN, CI, and CIV, with PMA treatment resulted in minimal cell adhesion and occasional PPF.

PKC activation with PMA is known to induce a series of biological processes related to differentiation correlating with the activation of MAPK.[43] In our study, activation of PKC by PMA in the absence of FN resulted in weak and rapid phosphorylation of ERK1/ERK2; however, cells increased only in size and ploidy, without either adhesion or PPF. While the polyploidy pattern is similar in the presence of PMA alone and of PMA/FN, only the

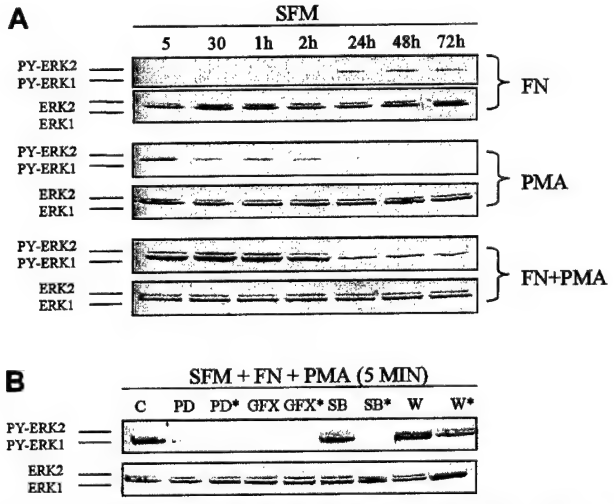


Figure 3. Correlation of ERK1/ERK2 MAPK activation with PPF. Cells were cultured in FN/PMA for 3 days and analyzed by Western blot. (A) Time course of ERK1/ERK2 phosphorylation in the cultures with the FN/PMA combination or with FN or PMA alone. The upper panels show that phosphorylation of ERK1/ERK2 was faintly expressed in the presence of FN alone after 1 day of culture. The middle panel shows that phosphorylation of ERK1/ERK2 was rapid and weakly expressed in the presence of PMA alone and diminished with time. The lower panel shows that phosphorylation of ERK1/ERK2 was strongly expressed only in the presence of FN plus PMA after 5 minutes' treatment and was sustained up to 3 days, correlating with PPF. (B) Effect of signal inhibitors on phosphorylation of ERK1/ERK2. The inhibitors were added 1 hour prior to addition of PMA for 5 minutes. The upper panel shows that phosphorylation of ERK1/ERK2 was inhibited by PD, an MEK inhibitor (10 μM and 50 μM*); GFX, a PKC inhibitor (5 μM and 25 μM*); and SB, a p38 and JNK MAPK inhibitor (50 μM*). No inhibition seen with SB (10 μM) and wortmannin (W), a PI3K inhibitor (100 nM and 500 nM*). SFM indicates serum-free medium. The data are representative of 3 separate experiments.

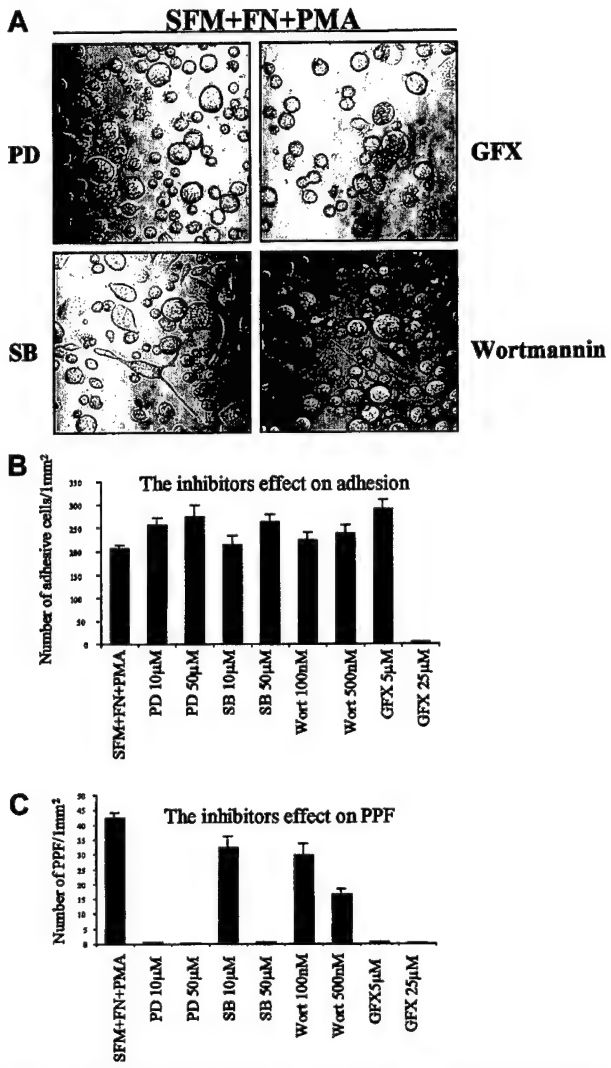


Figure 4. Effect of signal inhibition on cell morphology. Cells were cultured in serum-free medium in the presence of FN. Cells were pretreated with inhibitors for 1 hour at 37°C; then PMA (10 ng/mL) was added and culture proceeded for 3 days. (A) Morphologic observation. Cells were visualized and measured by means of phase contrast microscopy at original magnification × 200. Representative fields demonstrating the results of at least 10 separate experiments are shown. (B) Morphologic quantitation. Morphologic assay was carried out by means of a phase contrast microscope (see "Materials and methods"). The cells with PPF were defined as those bearing more than one cytoplasmic process that were at least twice the length of the cell body diameter. SFM indicates serum-free medium; PD, PD98059, an inhibitor of MEK MAPK (upstream of ERK); GFX, GF109203X, an inhibitor of PKC; SB, SB203580, an inhibitor of p38 and JNK; Wort, wortmannin, an inhibitor of PI3K. Graph depicts the means ± SE from at least 10 experiments.

latter induced adhesion and PPF. Thus, while polyploidy or adhesion is essential for PPF, neither alone is sufficient to induce this morphologic change. Only FN/PMA costimulation induced a strong, rapid, and sustained phosphorylation of ERK1/ERK2 that lasted for at least 72 hours and correlated with full differentiation, including adhesion, spreading, size increase, polyploidization, and PPF. Since similar results were obtained with either serum/PMA or FN/PMA, the integrin ligand FN appears to be the component in serum responsible for the activation process leading to cell differentiation and PPF. Even though VN can also bind to $\alpha v\beta_3$, $\alpha v\beta_5$, and $\alpha IIb\beta 3$, which are also receptors for FN, it could not induce full differentiation of CHRF cells with PMA treatment; neither did CI bound to $\alpha IIb\beta 3$. The strong ERK1/ERK2 phosphorylation with FN/PMA was due to an amplification process resulting from cross-talk between the integrin- and the PKC-generated signaling pathways.

Inhibitors of signaling pathways were used in order to clarify whether there was a causal relationship between ERK1/ERK2 activation and PPF. PD98059, an MEK inhibitor upstream of ERK1/ERK2, blocked the phosphorylation of ERK1/ERK2 as well as PPF, though polyploidy and adhesion were not affected. PPF therefore required not only adhesion to an FN matrix and polyploidy, but also sustained activation of ERK1/ERK2. A causal relationship was therefore demonstrated between ERK1/ERK2 activation and PPF.

The phenotypic differences between surface markers of differentiated and undifferentiated cells are related to the maturation process as reflected by the decrease of CD90 and CD33, early myeloid surface markers. The significant decrease of CD62P in differentiated cells may be due to its translocation to the PPF. CD62P is an α-granule protein in primary MKs and platelets that is expressed on the platelet surface following platelet activation. Its expression on the surface of CHRF cells is therefore not typical for a platelet-producing cell line.

Since adhesion and polyploidy are necessary, though not sufficient, for PPF, we investigated stimulation of MAPK pathways other than ERK1/ERK2. The JNK/SAPK pathway is usually activated in response to stress/apoptosis. In this study, phosphorylation of JNK1/SAPK, also induced by FN/PMA costimulation, appeared rapidly in concert with ERK1/ERK2 activation but disappeared after 24 hours, just when cells started to develop PPF. SB203580, an inhibitor of JNK1/SAPK, prevented JNK1/SAPK phosphorylation but did not affect differentiation features of CHRF cells or PPF, when used at a concentration of 10 μM. Thus, though JNK1/SAPK activation correlates with CHRF differentiation, it is not involved in PPF. At a high concentration (50 μM), SB203580 blocked PPF, probably via inhibition of ERK1/ERK2 (Figure 3B).

The p38 MAPK is activated when apoptosis, following differentiation, is triggered.[40] PI3K, on the other hand, is related to survival, growth, and differentiation.[32,39] Since p38 MAPK and PI3K have been reported in some studies to be upstream of the pathway leading to ERK1/ERK2 activation,[44,45] we investigated whether this was the case with CHRF cells. In this study, neither kinase was activated following treatment with the FN/PMA combination, and the inhibitors to the respective kinases did not affect cell differentiation or ERK1/ERK2 phosphorylation.

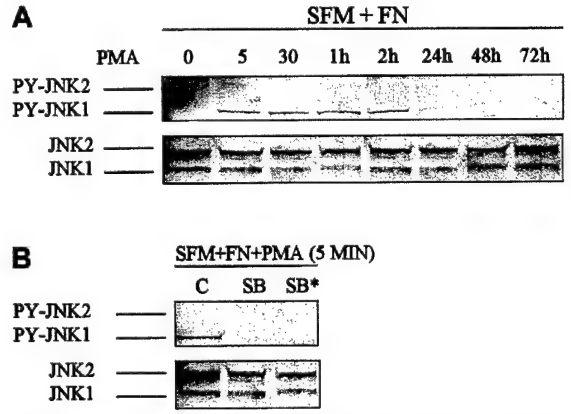


Figure 5. SAPK/JNK1 MAPK activation. Cells were cultured in FN/PMA for 3 days and analyzed by Western blot. (A) The upper panel shows that only phosphorylation of SAPK/JNK1 (46 kd) was expressed for up to 2 hours. (B) SB203580 (10 μM and 50 μM*), an inhibitor of SAPK/JNK added 1 hour prior to PMA treatment, blocked phosphorylation of SAPK/JNK1 (46 kd). SFM indicates serum-free medium. The data are representative of 3 separate experiments.

Recent reports have shown that TPO activated MK differentiation through the ERK MAPK pathway.[46] TPO was ineffective in promoting differentiation features in CHRF cells (data not shown), presumably owing to the lack of the c-Mpl receptor. Only stimulation with the nonphysiological agonist PMA in the presence of FN triggers differentiation.

The ultrastructure of PPF and released particles as well as the dysfunction of particles with platelet agonists demonstrates that the CHRF-288 cell line does not produce bona fide platelets. This transformed cell line, however, provides valuable information on the mechanism of MK differentiation, ploidy, and PPF and is useful for a better understanding of molecular events associated with MK development and platelet production.

Acknowledgments

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Appendix V

Isaac Cohen - DAMD-98-1-8327

METHODS IN MOLECULAR BIOLOGY™

Cytokines and Colony Stimulating Factors

Methods and Protocols

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Large-Scale Ex Vivo Expansion of Human Megakaryocytes for Clinical Use

Phil Lefebvre and Isaac Cohen

1. Introduction

This chapter will describe a method used to produce human megakaryocytes in vitro (*see* **Fig. 1**), in high yield, for a clinical product to supplement progenitor cell transplants. Producing significant numbers of megakaryocytes from human progenitor cells requires specific combinations of cytokines to promote their proliferation and maturation in vitro. Thrombopoietin (TPO) is the primary regulator of megakaryocytopoiesis *(1–6)*. Other cytokines, including interleukin-3 (IL-3), IL-6, IL-11, stem cell factor (SCF; also known as *kit*-ligand), and granulocyte–macrophage colony stimulating factor (GM-CSF) are also capable of promoting megakaryocytopoiesis and will synergize with TPO to increase megakaryocyte proliferation (reviewed in **ref. 7**).

Properly maintained (e.g., refed and split at regular intervals), static in vitro liquid cultures of purified human bone marrow CD34+ cells can take approx 2 wk for the progenitor cells to terminally differentiate and undergo apoptosis *(8)*. In the presence of TPO alone, these cultures can reach 90+% CD41+ (the primary surface marker for megakaryocytes), but the range of megakaryocyte production and maturation is highly variable and is dependent on culture conditions and the quality of starting material. Human umbilical cord blood CD34+ cells are capable of greater proliferation, but less capable of producing high-ploidy megakaryocytes. Human peripheral blood CD34+ cells perform similarly to bone marrow cells, although the quality of the patient's progenitor cells will ultimately determine productivity *(9)*. Adding other mitogenic cytokines, such as IL-3, will significantly decrease the frequency of CD41+

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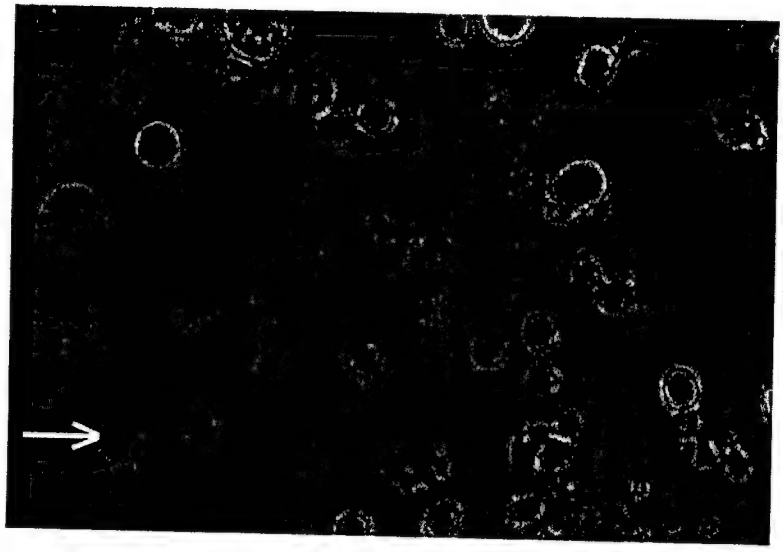

Fig. 1. Phase-contrast image of an 85% pure, 12-d human megakaryocyte culture. The white arrow points to a megakaryocyte that is beginning to form proplatelet extensions. The black arrow points to a megakaryocyte that has completed this process and shed platelets. Magnification = 400×.

cells, but will even more significantly increase the rate of cell expansion, resulting in a net increase in total megakaryocytes produced *(10)*. Although numerous cytokines can be combined to promote ex vivo expansion of megakaryocytes *(11)*, a significant expansion of megakaryocytes can be achieved with as little as two or three cytokines *(10,12–14)*, which may prove more practical in a clinical setting.

Unlike murine megakaryocytes, no culture condition has yet been shown to promote normal polyploidal nuclear maturation of human megakaryocytes in vitro, defined as a modal ploidy of 16 *N* or 8 pairs of chromosome sets. Also, there is an inverse relationship between proliferation and maturation of cultured human megakaryocytes *(12–15)*. Culturing with TPO alone, which is not a great promoter of cell proliferation, will generate a higher level of megakaryocyte maturation than when combined with more mitogenic cytokines, though a recent article has shown that stromal-cell-derived factor 1 can increase the number and ploidy of megakaryocytes derived in vitro from human CD34+ cells cultured in the presence of TPO at low cell concentrations under low oxygen tension *(16)*, although a normal 16N modal ploidy was still not achieved.

We and others *(10,11)* have found that serum-free medium can be more productive for ex vivo expansion than medium containing plasma. Serum-free

medium would also be preferable for ex vivo expansion of cells for transplant, because of the risks associated with allogeneic blood transfusions, and because it is a more consistent and well-defined product. Serum derived from clotted blood is not appropriate for promoting megakaryocyte expansion, as it contains platelet-derived factors inhibitory to megakaryocyte growth, such as transforming growth factor β, β-thromboglobulin, and platelet factor 4, all released from activated platelets *(17–19)*. If cost is a factor, autologous, heparinized platelet-free plasma or autologous serum derived from calcium-clotted platelet-free plasma could also be suitable for culturing megakaryocytes, as it eliminates the risk associated with allogeneic blood transfusion.

2. Materials

2.1. Media, Buffers, and Cytokines

1. Culture medium: X-vivo 20 serum-free medium (BioWhittaker, Walkersville, MD) (*see* **Note 1**).
2. Thawing medium: X-vivo 20, containing 10 IU preservative-free heparin and 10 µg/mL of DNase.
3. Growth factors used (final concentration): TPO (100 ng/mL), IL-3 (10 ng/mL), Flt-3 ligand, (Flt-3L, 100 ng/mL). Aliquots of each reconstituted cytokine are stored at –70°C until needed. Thawed aliquots can be kept at 4°C for up to 2 wk.
4. Phosphate-buffered saline (PBS), without calcium or magnesium.
5. Hypotonic citrate; 1 mg/mL of sodium citrate in deionized water.
6. DNA dye; propidium iodide or 7-aminoactinomycin D (7-AAD).

2.2. CD34+ Cell Selection

Miltenyi MACS CD34+ selection system appropriate for the quantity of cells used.

2.3. Colony Assays

Needed if the primitive progenitor population is of interest. We have tested several different clonogenic assay protocols and have found the kits from Stem Cell Technologies (Vancouver, BC, Canada) for CFU-MK (MegaCult C) and CFU-GM (MethoCult) to be the most efficient.

2.4. Quantitative Cell Analysis

1. 1% Trypan blue (Sigma) in saline, for cell enumeration and determination of viability.
2. Anti-human CD34 and CD41 antibodies, coupled to different fluorochromes, for flow-cytometric analysis. A comparable isotypic antibody, labeled with the same fluorochrome, is used as a negative control.

2.5. Instrumentation

1. 37°C Water bath.
2. Class B sterile tissue culture hood.
3. Clinical bench centrifuge.
4. 37°C tissue culture incubator at 5% CO_2.
5. Variable adjustable electronic pipettor, EDP, Rainin.
6. Vacuum-driven automatic pipet device.

2.6. Supplies

1. Sterile pipet tips with aerosol barrier.
2. Sterile individually wrapped polypropylene transfer pipets.
3. 2-mL, 5-mL, 10-mL, 25-mL Sterile individually wrapped polystyrene pipets with an aerosol barrier.
4. Sterile centrifuge tubes.
5. 1-cm^3, 3-cm^3, 10-cm^3, 20-cm^3, 30-cm^3, 60-cm^3 sterile Luer-Lok™ syringes.
6. VueLife Teflon tissue culture bags, 1 L (270-mL nominal capacity) and 7-mL capacity, American Fluoroseal Corporation. Other sizes are also available, including custom sizes.
7. Luer-Lok connector tubing, available from American Fluoroseal Corp.
8. Sterile threaded Luer-Lok cannulas (BD Interlink System; Becton Dickinson product no. 303369).
9. Interlink Luer-Lok injection site (Baxter Healthcare product no. 2N3379).

3. Method

3.1. CD34 Cell Selection

For large-scale clinical use, the CliniMACS clinical-grade system is preferred. It is approved for clinical use in the European Community, but is presently under evaluation for clinical use in the United States. Smaller-scale systems are available for preclinical or basic research use. The manufacturer-supplied laboratory protocol is straightforward and easy to follow and should be followed closely to ensure successful CD34 cell purification. Purity of over 90% with high yield (70+%) is common with all MACS systems, when used before the expiration date.

3.2. Sample Thawing

If cells were frozen after purification for storage and use at a later date, then they will need to be carefully thawed, as freezing and thawing can damage cells.

1. Each frozen CD34+ cell unit is placed into a resealable plastic bag and the bag closed, to prevent any contaminating water seepage into the tube.

2. The sealed bag with the tube is then placed in a 37°C water bath and gently agitated until the cell suspension has thawed 90%, to a small ball of ice (approx 3–4 min for a 5-mL tube).
3. The bag is wiped dry and transferred to a Class B sterile tissue culture hood, where the tube is removed from the bag aseptically. (*See* **Notes 2–4.**)

3.3. Sample Washing

1. In the sterile tissue culture hood, the contents of a 5-mL tube of thawed cells is transferred by gentle addition into a sterile 50-mL centrifuge tube using a sterile polypropylene transfer pipet.
2. The cells are diluted 1 : 10 with 45 mL of cold thawing medium.
3. The tube is sealed and the cells gently mixed.
4. The suspension is centrifuged at 260*g* for 10 min at 4°C.
5. The cell pellet is resuspended in 3 mL of X-vivo 20 using a sterile polypropylene transfer pipet.
6. Dilute the cell suspension to 50 mL with X-vivo 20 (not thawing medium) by decanting fresh medium.
7. The cell suspension is centrifuged again at 260*g* for 10 min at 4°C.
8. The cell pellet is resuspended in 3 mL of X-vivo 20 using a sterile polypropylene transfer pipet.

3.4. Nucleated Cell Count

1. A 50-µL sample for cell concentration analysis is taken using a sterile pipet tip with an aerosol barrier.
2. The sample is diluted with trypan blue so that the cell concentration in the trypan blue is not more than 10^6 cells/mL.
3. Apply 10–12 µL of trypan blue cell suspension to a hemacytometer.
4. Determine the cell concentration and viability (cells that appear blue are not viable) using a phase-contrast microscope.
5. The final volume required to achieve a cell concentration of 400,000 cells/mL is calculated, based on the cell count.

3.5. Culture Preparation

1. The cells are injected using a syringe connected to a Luer-Lok cannula, through the Interlink injection port, into a Teflon bag of appropriate size (*see* **Fig. 2**).
2. The bag's nominal volume is adjusted with bag clip barriers so as to contain a volume sufficient to suspend the cells at 400,000 cells/mL at an average fluid height of 1 cm.
3. X-vivo 20 and sufficient cytokines to achieve a final concentration of 100 ng/mL Flt-3L, 100 ng/mL TPO, and 10 ng/mL IL-3 is then added using a 60-cm^3 syringe attached via Luer-Lok connections to the bag to reach the final volume. (*See* **Note 5.**)

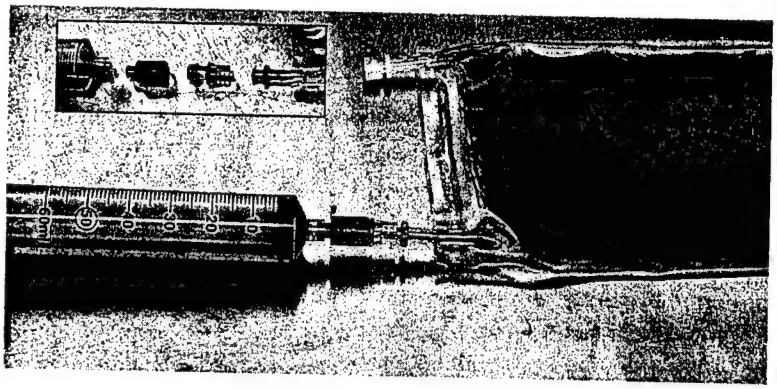

Fig. 2. Syringe–bag connection system. Inset: exploded view showing (from left to right) Luer-Lok 60-cm^3 syringe, Interlink threaded lock cannula, Interlink injection site, and Luer-Lok opening of Teflon bag.

3.6. Culture Maintenance

Cells should be assayed at least every other day to ensure that they have not proliferated beyond the capacity of the culture medium. In our experience, X-vivo 20 has a maximum cell capacity of up to 2×10^6 cells/mL, depending on the quality of the cells used.

1. After gently agitating the bag to resuspend the cells, a 5-mL aliquot is removed using a syringe and injected into a 7-mL Teflon bag for use as a test sample.
2. Both bags are stored in a 37°C tissue culture incubator at 5% CO_2 used solely for the purpose of clinical patient sample preparation. (*See* **Notes 6–8.**)

3.7. Refeeding the Culture

When cells have proliferated beyond the culture medium's limit, they will need to be split and refed.

1. Count the cells as in **Subheading 3.4.**
2. Calculate the amount of medium and cytokines required to readjust the cell concentration to 400,000 cells/mL.
3. Calculate the area required to maintain a 1-cm culture height and readjust the bag clips to that position.
4. Add that amount to the culture bag using the Luer-Lok syringes and cannula connectors.
5. If the culture medium required exceeds the capacity of the bag, additional bags are then attached to the culture bag using the Luer-Lok connection tubing, and an appropriate fraction of cell suspension is transferred to the new bag (*see* **Fig. 3**). (*See* **Note 9.**)

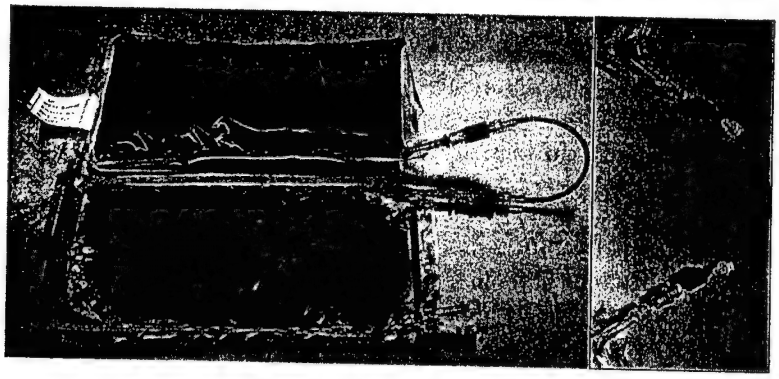

Fig. 3. Bag–bag connection system. Inset: Exploded view. A flexible tube with Luer-Lok connectors on each end is substituted for the syringe in **Fig. 2.**

3.8. Cell Harvest

When the designated culture period has passed, the sample is transferred to an appropriate vessel and gently washed, PBS with 1% albumin, or other clinically approved procedure. Cells can be washed in an automated cell washer or centrifuged using centrifuge tubes or bottles of sufficient capacity to contain the culture. The appropriate procedure needs to be determined empirically, based on culture size, equipment available, and whether a closed system is required for clinical use. Three wash steps should be used ensure that no residual cytokine remains in any product designated for transplant. (*See* **Note 10.**)

3.9. Cell Analysis

Flow cytometry is the most efficient way of measuring phenotype expression of cells in a liquid culture. Megakaryocytes can be difficult to assay, however, because of their fragility and great variation in size. Cells that may have been exposed to platelets (e.g., peripheral blood cells, umbilical cord cells, or cells in platelet-producing megakaryocyte cultures) should be incubated in EDTA-containing buffer to disassociate any adherent platelets *(20)*. Then, standard flow-cytometric techniques can be used to assay megakaryocyte proliferation. (*See* **Note 11.**)

1. Place cell medium containing 100,000 cells into a 12 × 75-mm test tube, appropriate for use on a flow cytometer.
2. Add 2 m*M* EDTA and incubate for at least 15 min at room temperature. Longer incubations may be necessary if platelet contamination is high.
3. Fill the tube with PBS and centrifuge at 260*g* for 5 min at 4°C.

4. Remove all supernatant and add the recommended amount of fluorochrome-labeled antibody. Vortex gently.
5. Incubate for 15 min at 4°C in the dark.
6. Fill the tube with PBS and centrifuge at 260*g* for 5 min at 4°C.
7. Remove supernatant, resuspend the cells in 0.5–1 mL of PBS and assay by flow cytometry immediately.
8. If megakaryocyte ploidy measurement is desired, substitute 1 mL of hypotonic citrate buffer for the PBS. Add 1 µg/mL of propidium iodide or 7-AAD. Incubate an additional 30 min at room temperature before assaying.

4. Notes

1. We have tested numerous serum-free media and have found X-vivo 20 to be the most conducive to megakaryocyte proliferation. However, technology can continue to evolve, so it would be expected that improved media should become available in the future. Further testing would be warranted to find the medium optimal for each protocol.
2. It is our experience that mononuclear cells are more fragile during freezing and thawing than selected CD34 cells. In either case, DNase is recommended for the thawing medium to remove DNA released from lysed cells. However, work within the recommended levels, as too much DNase can be harmful to cells.
3. We and others *(21)* have found the Miltenyi CD34+ MACS selection systems to be the most efficient. The systems available range from a total cell capacity of 10^5–10^{11} cells. We have used several different Miltenyi systems, all with excellent results.
4. The Fluoroseal Teflon bags are impermeable to liquid, but permeable to gas. This allows adequate gas exchange while all ports remain fully sealed, so that vapor cannot escape the bag. What this means is that liquid medium loss is not a problem, so it is not necessary to keep water in the incubator for humidity, reducing contamination risk.
5. For a 1-L Teflon bag, only 270 mL is used so that the culture medium height remains at approx 1 cm, which has been determined to be optimal for culture performance. Lower heights reduce cell productivity, whereas higher media heights waste reagent. The use of bag clips allows for volumes less than 270 mL to be used in the 1-L bags, and then removed as the culture expands and is refed. American Fluoroseal supplies appropriate clips, but any plastic clip that would not damage the plastic bags would work.
6. It has been our experience that ex vivo cultured cells derived from oncology patients will often show a dramatic drop in viable cell numbers in the first few days of a culture. This phenomenon is true whether the cells were unselected mononuclear cells or selected CD34 cells and it is highly variable.
7. The use of a smaller, 7-mL bag is to be able to monitor culture progress without needing to access the main culture, reducing the risk of contamination. In our experience, the phenotypic development of the cells in either bag is identical.

8. We have found that megakaryocyte proliferation is enhanced with an initial seeding density of 400,000 CD34 cells/mL, compared to lower seeding densities. The culture conditions described have a total cell capacity in the range of up to 2×10^6 cells/mL (fivefold expansion) before having a significant negative impact on cell proliferation and viability. However, cell concentration may need to be readjusted after only a twofold to threefold expansion of viable cells to minimize any negative effects of large numbers of nonviable cells. This is most often in cases of less healthy or damaged cell samples that rapidly degenerate into nonviable states.
9. The easiest way to transfer expanded cells from on bag to another is by attaching the two bags together with a Luer-Lok connector tube and suspending the full bag so that medium runs down into the empty bag. When the cell culture appears to be equally distributed in each bag, they should be weighed and equilibrated by weight. This avoids excessive opening and taping of the bags, reducing contamination risk.
10. Once mature megakaryocytes are generated, care should be taken in handling the culture, as large high-ploidy megakaryocytes are fragile. Avoid extremes of handling and vortexing, if preservation of the larger, more mature megakaryocytes is important.
11. Although there are many procedures for fixing and permeabilizing megakaryocytes, the method using hypotonic citrate is the easiest and best preserves the large ploidy megakaryocytes, because of significantly less manipulation.

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